PATENT
Docket No.: **19603/2986 (CRF D-1940B)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Qiu et al.	Examiner: Anne R. Kubelik
Serial No.	:	09/766,348	
Cnfrm. No.	:	7683	Art Unit: 1638
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For	:	HYPERSENSITIVE RESPONSE INDUCED RESISTANCE IN PLANTS BY SEED TREATMENT	

RESPONSE TO NOTIFICATION OF NON-COMPLIANT APPEAL BRIEF

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Notification of Non-Compliant Appeal Brief, dated April 28, 2008, applicants hereby submit a revised Evidence Appendix as required by 37 C.F.R. § 41.37(c)(1)(ix), as follows:

X. EVIDENCE APPENDIX

A. EXHIBIT 1 - Koncz et al., "The Opine Synthase Genes Carried by Ti Plasmids Contain All Signals Necessary for Expression in Plants," *EMBO J.* 2(9):1597-1603 (1983)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

B. **EXHIBIT 2 -** U.S. Patent No. 5,034,322 to Rogers et al.

- Discussed in appellants' Amendment Under 37 CFR § 1.116, dated March 29, 2005, and considered by the examiner in the Advisory Action, dated April 20, 2005.

C. **EXHIBIT 3 -** U.S. Patent No. 5,352,605 to Fraley et al.

- Discussed in appellants' Amendment Under 37 CFR § 1.116, dated March 29, 2005, and considered by the examiner in the Advisory Action, dated April 20, 2005.

D. **EXHIBIT 4 -** U.S. Patent No. 5,850,015 to Bauer et al.

- Relied upon and discussed in the Examiner's Answer, dated November 20, 2006.

E. **EXHIBIT 5 -** U.S. Patent No. 6,174,717 to Beer et al.

- Relied upon and discussed in the Examiner's Answer, dated November 20, 2006.

F. **EXHIBIT 6 -** Tampakaki et al., "Elicitation of Hypersensitive Cell Death by Extracellularly Targeted $HrpZ_{Psph}$ Produced In Planta," *Molecular Plant-Microbe Interactions* 13:1366-1374 (2000)

- Relied upon and discussed in the Examiner's Answer, dated November 20, 2006.

G. **EXHIBIT 7 -** Declaration of Zhong-Min Wei Under 37 C.F.R. § 1.132 ("Second Wei Declaration")

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

H. **EXHIBIT 8** – Gopalan et al., "Bacterial Genes Involved in the Elicitation of Hypersensitive Response and Pathogenesis," *Plant Disease* 80:604-10 (1996)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

I. **EXHIBIT 9** – Bauer et al., "*Erwinia chrysanthemi* Harpin$_{Ech}$: An Elicitor of the Hypersensitive Response that Contributes to Soft-Rot Pathogenesis," *MPMI* 8(4):484-91 (1995)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

J. **EXHIBIT 10** – Cui et al., "The RsmA$^-$ Mutants of *Erwinia carotovora* subsp. c*arotovora* Strain Ecc71 Overexpress *hrpN*$_{Ecc}$ and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," *MPMI* 9(7):565-73 (1996)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

K. **EXHIBIT 11** – Ahmad et al., "Harpin Is Not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," *8th Int'l. Cong. Molec. Plant-Microbe Inter.* July 14-19, 1996

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

L. **EXHIBIT 12** – Jock et al., "Molecular Differentitation of *Erwinia amylovora* Strains from North America and of Two Asian

Pear Pathogens by Analyses of PFGE Patterns and *hrpN* Genes," *Environ. Microbiol.* 6(5):480-490 (2004)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

M. **EXHIBIT 13** – Preston et al., "The HrpZ Proteins of *Pseudomonas syringae* pvs. s*yringae, glycinea,* and *tomato* Are Encoded by an Operon Containing *Yersinia ysc* Homologs and Elicit the Hypersensitive Response in Tomato but not Soybean," *MPMI* 8(5):717-32 (1995)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

N. **EXHIBIT 14** – Bonas, "*hrp* Genes of Phytopathogenic Bacteria," *Current Topics in Microbiology and Immunology* 192:79-98 (1994)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

O. **EXHIBIT 15** – Alfano et al., "The Type III (Hrp) Secretion Pathway of Plant Pathogenic Bacteria: Trafficking Harpins, Avr Proteins, and Death," *Journal of Bacteriology* 179:5655-5662 (1997)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

P. **EXHIBIT 16** – Swanson et al., "Isolation of the *hreX* Gene Encoding the HR Elicitor Harpin (Xcp) from *Xanthomonas campestris* pv. p*elargonii*," *Phytology* 90:S75 (1999)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

Q. **EXHIBIT 17** – Bogdanove et al., "Unified Nomenclature for Broadly Conserved *hrp* Genes of Phytopathogenic Bacteria," *Molec. Microbiol.* 20:681-683 (1996)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

R. **EXHIBIT 18** – Wei et al., "Regulation of *hrp* Genes and Type III Protein Secretion in *Erwinia amylovora* by HrpX/HrpY, a Novel Two-Component System, and HrpS," *MPMI* 13(11):1251-1262 (2000)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

S. **EXHIBIT 19** – Bonas, "Bacterial Home Goal by Harpins," *Trends Microbiol* 2:1-2 (1994)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

T. **EXHIBIT 20** – WO 01/98501 to Fan et al.

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

U. **EXHIBIT 21** – Wei et al., "Harpin from *Erwinia amylovora* Induces Plant Resistance," *Acta Horticulturae* 411:223-225 (1996)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

V. EXHIBIT 22 – Strobel et al., "Induction of Systemic Acquired Resistance in Cucumber by *Pseudomonas syringae* pv. *syringae* 61 $HrpZ_{Pss}$ Protein," *Plant Journal* 9(4):431-439 (1996)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

W. EXHIBIT 23 – U.S. Patent Application Publication No. 2004/0073970 to Takakura et al.

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

X. EXHIBIT 24 – Lund et al., "A Plant Signal Sequence Enhances the Secretion of Bacterial ChiA in Transgenic Tobacco," *Plant Mol. Biol.* 18:47-53 (1992)

- Introduced into the record by appellants on August 13, 2004, and considered by the examiner in the office action dated October 29, 2004.

Also submitted herewith are copies of Exhibits 8-24 of the above revised Evidence Appendix. (Exhibits 1-7 were provided with the Appeal Brief submitted on January 28, 2008.)

Respectfully submitted,

Date: May 28, 2008

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Bacterial Genes Involved in the Elicitation of Hypersensitive Response and Pathogenesis

Intensive molecular genetic studies undertaken in the past 10 years have started to solve many of the puzzles in the area of compatibility and incompatibility between plants and bacterial pathogens. These studies have provided answers to some of the most fundamental questions in plant pathology: What bacterial genes are involved in the establishment of compatibility or incompatibility between plants and necrogenic bacteria? What traits distinguish plant-pathogenic bacteria from saprophytic bacteria? Are these genes and traits common in seemingly very diverse groups of plant-pathogenic bacteria, from soft-rot erwinias to local lesion-forming pseudomonads? In this article, we will discuss some recent advances in understanding the compatibility or incompatibility between plants and necrogenic bacteria (bacteria that cause tissue necrosis). The potential application of these advances to disease management will be addressed briefly. Interested readers should consult other recent reviews (6,8,45,50) for a more technical discussion on this topic.

Plant–Bacteria Interactions: Incompatible vs. Compatible

Plant-pathogenic bacteria cause devastating diseases on many important crop plants. Some bacteria, such as *Agrobacterium tumefaciens*, cause tissue deformation (tumors) by altering hormone balance in infected plant tissues. Other bacteria cause wilt or soft rot by interfering with the function of the plant vascular system or by disintegrating plant tissues, respectively. Many pathovars of *Pseudomonas syringae* and *Xanthomonas campestris* cause local lesions on various plant tissues. Disease symptoms caused by most plant-pathogenic bacteria involve plant cell death. In this article, only necrogenic bacteria will be discussed. Therefore, gall-forming *A. tumefaciens* and other bacteria that do not cause necrosis will not be addressed.

Plant–bacteria interactions can be generally classified as compatible or incompatible interactions. In a compatible interaction, a susceptible host plant is infected by a virulent (or compatible) bacterium, resulting in the multiplication and spread of the bacterium in infected plant tissues and the appearance of disease symptoms. In an incompatible interaction, an avirulent (or incompatible) bacterium attempts to infect a resistant host plant or a nonhost plant, but the multiplication and spread of the bacterium are severely restricted. A hallmark of many incompatible interactions is the occurrence of rapid plant cell death at or near the attempted infection site, a phenomenon known as the hypersensitive response (HR; 16,29). That is, although an avirulent bacterium is unable to cause typical spreading disease symptoms in a resistant host or nonhost plant, it is able to elicit localized plant cell death. The HR is associated with a wide array of defense responses that may inhibit further pathogen invasion, including synthesis of antimicrobial compounds, induction of plant defense genes, and strengthening of the plant cell wall by rapid cross-linking of cell wall components (10,32).

Although a true plant-pathogenic bacterium can elicit a dramatic plant response—either disease or resistance—in a healthy plant with the appropriate genetic background, saprophytic bacteria or bacteria that are pathogenic on organisms other than higher plants are generally unable to initiate any interactions in plants. Of 1,600 known species of bacteria, only about 80 species have been found to cause plant disease (1). What are the features that distinguish plant-pathogenic bacteria from other types of bacteria? Taxonomic differences give no clue to the differences in pathogenicity. For example, *Erwinia amylovora*, the bacterium that causes fire blight, is taxonomically more closely related to the human pathogens *Escherichia coli* and *Yersinia* spp. than to another common plant pathogen, *P. syringae*.

Genes Controlling Compatibility Between Plants and Bacteria

In the early 1980s, a number of researchers started to use transposon-mediated mutagenesis, a technique developed in the study of *E. coli*, to reveal bacterial genes that play important roles in various plant–bacteria interactions. A transposon is a mobile DNA element that can hop in and out of the bacterial chromosome. When a transposon hops into a gene on the chromosome, the gene is physically disrupted and cannot produce a functional product (Fig. 1). If the gene happens to be important in plant–bacterial interactions, the mutant bacterium carrying the disrupted gene will show a defect in initiating normal plant–bacterial interactions.

Using such a mutagenesis technique, Niepold et al. (35) and Lindgren et al. (33) identified clusters of bacterial genes, known as *hrp* (for HR and pathogenicity) genes, in the bean pathogens *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *phaseolicola*, respectively. Transposon-induced mutations in *hrp* genes were found to abolish the ability of *P. syringae* to elicit the HR in nonhost plants or to cause disease in host plants (33,35). *hrp* mutants behave very much like bacteria that have no apparent interactions with plants, such as *E. coli*. The identification of *hrp* genes suggested that the molecular mechanism(s) underlying bacterial pathogenicity and bacterial elicitation of plant disease resistance may involve the same bacterial genes.

hrp genes have been isolated from many plant-pathogenic bacteria and have been characterized most extensively from *P. s.* pv. *syringae*, *P. s.* pv. *phaseolicola*, *Pseudomonas solanacearum* (which causes wilt in many solanaceous plants), *Xanthomonas campestris* pv. *vesicatoria* (which causes bacterial spot on tomato and pepper), and *E. amylovora* (6,8,45). Isolation (cloning) of *hrp* genes was accomplished by inserting random genomic DNA fragments from a wild-type, plant-pathogenic bacterium into a cloning vector, followed by introduction of cloned DNA fragments into *hrp* mutants

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(Fig. 1). If a cloned DNA fragment carries a wild-type copy of the mutated *hrp* gene in an *hrp* mutant, it will produce a functional *hrp* gene product and therefore complement the mutated *hrp* gene located in the chromosome (Fig. 1). Surprisingly, the cloned *hrp* clusters from *P. s.* pv. *syringae* 61 and *E. amylovora* 321 enabled nonpathogens (e.g., *E. coli* or *Pseudomonas fluorescens*) to elicit the HR in plants (5,24). The functional cloning of these two *hrp* clusters in *E. coli* revealed that the minimum number of genes required for elicitation of the HR by plant-pathogenic bacteria is carried on a DNA fragment about 25 to 30 kb in length, a very small portion of the bacterial genome (which is normally about 4,000 to 5,000 kb).

DNA–DNA hybridization studies indicate that at least some *hrp* genes are similar among necrogenic bacteria belonging to different genera (*P. syringae, E. amylovora, Erwinia stewartii, P. solanacearum,* and *X. campestris*) (31). Recent DNA sequence studies confirm that many *hrp* genes cloned from diverse plant-pathogenic bacteria are homologous (23,46). Thus, *hrp* genes appear to be universal among diverse necrosis-causing, gram-negative bacterial pathogens of plants.

Biochemical Functions of *hrp* Genes

The biochemical functions of *hrp* genes have remained a puzzle until recently. DNA sequencing has played a major role in the determination of many *hrp* gene functions. As will be discussed, many *hrp* genes have striking similarities with genes of known function. Figure 2 shows the gene organization and likely functions of *hrp* genes of *P. s.* pv. *syringae* (23). There are at least 25 *hrp* genes in this bacterium. Based on DNA sequence similarity to other known genes and subsequent biochemical and molecular characterization, we now know that *hrp* genes have at least three biochemical functions: gene regulation, protein secretion, and production of HR elicitor proteins.

1. Gene regulation. It was discovered that *hrp* genes either are not expressed or are expressed at very low levels (i.e., they make very low levels of protein products) when bacteria were grown in nutrient-rich bacteriological media, whereas they are highly expressed when bacteria are in the intercellular space (apoplast) of plant tissues (25,37,41,46,48,52,53). What conditions in plant tissues induce the expression of *hrp* genes, and how do bacteria detect these inducing conditions? Unlike viruses, nematodes, and many fungi, plant-pathogenic bacteria do not invade living plant cells. Therefore, signal exchanges between plant cells and bacteria must occur in (or through) the apoplast outside the plant cell. A number of laboratories have observed that induction of *P. syringae hrp* genes could be achieved by using artificial minimal media lacking complex nitrogen nutrients, indicating that lack of nutrients in the plant apoplast may be the signal for the induction of *hrp* genes (25,37,52,53). Specific compounds (e.g., sucrose and sulfur-containing amino acids) present in the plant apoplast may also serve as signals for the induction of *X. c.* pv. *vesicatoria hrp* genes (41). The induction of *hrp* genes in the nutrient-poor plant apoplast or in artificial minimal media indicates that *hrp* genes may be involved in bacterial nutrition in planta.

How do bacteria sense the plant apoplast environment? It was found that at least three of the 25 *hrp* gene products are involved in detection of the apoplast environment by *P. syringae*: *hrpL*, *hrpS*, and *hrpR* (18,51; Fig. 2). The *hrpS* and *hrpR* are among the first two *hrp* genes to be expressed once bacteria enter plant tissues (51,52). It has been hypothesized that the HrpS and HrpR proteins, once produced, bind to the "promoter" sequence of the *hrpL* gene to "promote" the production of the *HrpL* protein (51). Once the HrpL protein is produced, it activates promoters of other *hrp* genes and some bacterial avirulence (*avr*) genes, which determine gene-for-gene interactions between bacteria and plants (25,26,38,40,51; Fig. 3). Not all bacterial *avr* genes are regulated by *hrp* genes (30). Interestingly, *hrpS* and *hrpR* are similar in sequence to a family of bacterial proteins that regulate genes involved in diverse metabolic functions, including genes involved in nutrient transport and metabolism (18,51). The sequence similarity of *hrpS* and *hrpR* with gene regulators involved in nutrition appears to support the hypothesis that *hrp* genes are involved in bacterial nutrition in the nutrient-poor plant apoplast. This hypothesis is further supported by the observation that the expression of *hrp* genes can be turned off by complex nitrogen sources, tricarboxylic acid cycle intermediates, high osmolarity, and neutral pH, some of which are characteristic of rich bacterial media (25,37,41,46,52,53).

An *hrpS* homolog has been found in a very different bacterium, *E. amylovora* (S. V. Beer, *personal communication*). In *P. solanacearum*, a different *hrp* gene (*hrpB*) was found to be involved in the detection of the plant apoplast (15). Thus, different bacteria may or may not use the same mechanism to detect the apparently similar environment in the plant apoplast.

2. Protein secretion. One surprising finding from the sequence analysis of *hrp* genes was that many *hrp* genes show striking similarities to those involved in the secretion of proteinaceous virulence factors in human and animal pathogenic bacteria (12,17,22,39,46). Most plant-pathogenic

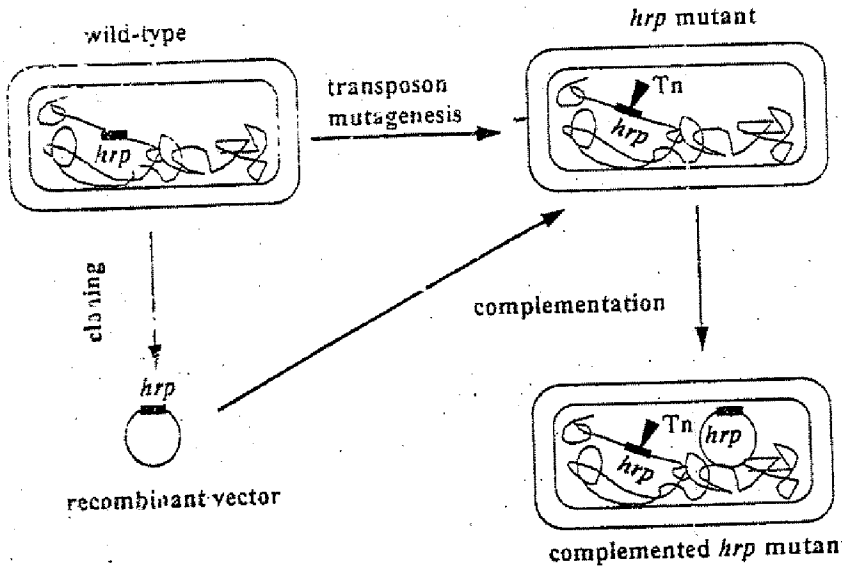

Fig. 1. Diagram of molecular techniques commonly used in the cloning of *hrp* genes. A wild-type bacterium is mutagenized by random insertion of a transposon (Tn) into its genome. When a transposon inserts into a wild-type *hrp* gene (in red), it physically disrupts the *hrp* gene (in green). The transposon-inserted *hrp* gene cannot produce a functional product, and the resulting bacterium is called a *hrp* mutant. The *hrp* mutant can no longer induce the hypersensitive response (HR) in resistant plants or cause disease in susceptible plants. To isolate (clone) the *hrp* gene identified by transposon mutagenesis, a gene library is established by inserting pieces of the wild-type genomic DNA into a cloning vector (indicated by a circle). The vector carrying foreign DNA inserts (recombinant vector) is then introduced into the *hrp* mutant. If a recombinant vector happens to carry a wild-type copy of the mutated *hrp* gene, it will produce a functional *hrp* gene product lacking in the *hrp* mutant, thus recovering the ability of the mutant to induce the HR in resistant plants and to cause disease in susceptible plants. The *hrp* mutant phenotype is therefore complemented by this recombinant vector.

bacteria that cause necrosis are gram-negative, that is, they have two cell membranes enveloping the cytoplasm. For any large molecule (e.g., a protein) to go through a lipid membrane, a special secretion apparatus or channel composed of many proteins must be assembled across both cell membranes. Gram-negative plant pathogenic bacteria are known to make several types of secretion apparatus. For example, *Erwinia chrysanthemi*, a bacterium that causes soft rot, makes one type of secretion apparatus for proteases and another for plant cell wall–degrading enzymes (21,39). Both types of secretion apparatus are widely conserved among many other bacteria, including human pathogens such as *E. coli* and *Pseudomonas aeruginosa* (21,39). The *hrp* genes were found to specify a third type of secretion apparatus, the Hrp secretion apparatus, which appears to be similar to the one discovered in several human-pathogenic bacteria, including *Yersinia* spp. (12,17,22,39,46). Interestingly, although the regulatory *hrp* genes in different bacteria may be different (*hrpS, hrpR*, and *hrpL* in *P. syringae* versus *hrpB* in *P. solanacearum*), most *hrp* genes involved in the assembly of the Hrp secretion apparatus are similar among diverse plant-pathogenic bacteria. This suggests that although different bacteria may detect the plant apoplast environment in their own unique ways, they nevertheless produce similar types of protein secretion apparatus.

3. Production of elicitor proteins. The discovery of the novel Hrp secretion apparatus raised an immediate question: What are the proteins that traverse it? Since *hrp* genes are essential for bacteria, both to elicit the plant HR and to cause disease, it was expected that some of the proteins that traverse the Hrp secretion apparatus may be elicitors of plant HR and that others may be involved in causing necrosis during pathogenesis. Wei et al. (47) first provided evidence that one of the *E. amylovora hrp* genes (*hrpN*) encodes a proteinaceous elicitor (harpin). Harpin elicits HR necrosis when injected into the apoplast of appropriate plants (47). Although no *hrpN* gene homolog could be found in *P. syringae*, another proteinaceous HR elicitor ($harpin_{Pss}$) was identified and was shown to be encoded by a different *hrp* gene, *hrpZ* (20,36). Furthermore, $harpin_{Pss}$ was the first extracellular protein shown to be secreted via the Hrp secretion apparatus (20). A third bacterial protein elicitor of the HR was identified in *P. solanacearum* and was named PopA1 (2). The *E. amylovora* harpin, *P. s.* pv. *syringae* 61 $harpin_{Pss}$, and *P. solanacearum* PopA1, although largely dissimilar in primary sequences, share similar properties that may be important in their HR elicitor activities. They are all heat stable, glycine rich, and hydrophilic. Homologs of *E. amylovora* harpin and *P. s.* pv. *syringae* 61 $harpin_{Pss}$ have been identified in other pathogenic strains that belong to the genus *Erwinia* and the species *P. syringae*, respectively (4,20). Thus, at least three proteins that traverse the Hrp secretion apparatus of three diverse bacteria elicit the HR.

The Search for Proteins that Traverse the Hrp Apparatus

As mentioned earlier, bacterial mutants defective in the Hrp secretion apparatus are unable to elicit the HR in resistant plants and to cause disease in susceptible plants. The question is, how many proteins are secreted via the Hrp secretion apparatus? If harpins and PopA are the only proteins that traverse the Hrp secretion apparatus, then mutations in the genes that make harpins and PopA would also eliminate the ability of bacteria to elicit the HR in resistant plants and to cause disease in host plants. However, if there are other pathogenicity- or HR-related proteins secreted via the Hrp apparatus, mutations in only harpin- or PopA-encoding genes would not completely abolish the ability of bacteria to elicit the HR in resistant plants or to cause disease in host plants. Wei et al. (47) reported that mutations in the gene coding for harpin of *E. amylovora* destroyed the ability of the bacteria both to trigger the HR in resistant nonhost tobacco and to cause disease in susceptible pear fruits. Mutations in the gene coding for $harpin_{Ech}$ of *E. chrysanthemi* prevented the bacterium from triggering the HR in the nonhost tobacco and reduced the ability of the bacterium to initiate disease lesions in host plants (4). In the case of $harpin_{Pss}$ of *P. syringae*, mutation analysis has been complicated by the complex gene structure and organization surrounding the *hrpZ* gene. Conclusive data regarding the role of $harpin_{Pss}$ in plant–*P. syringae* interactions are yet to be shown. PopA1 was shown to

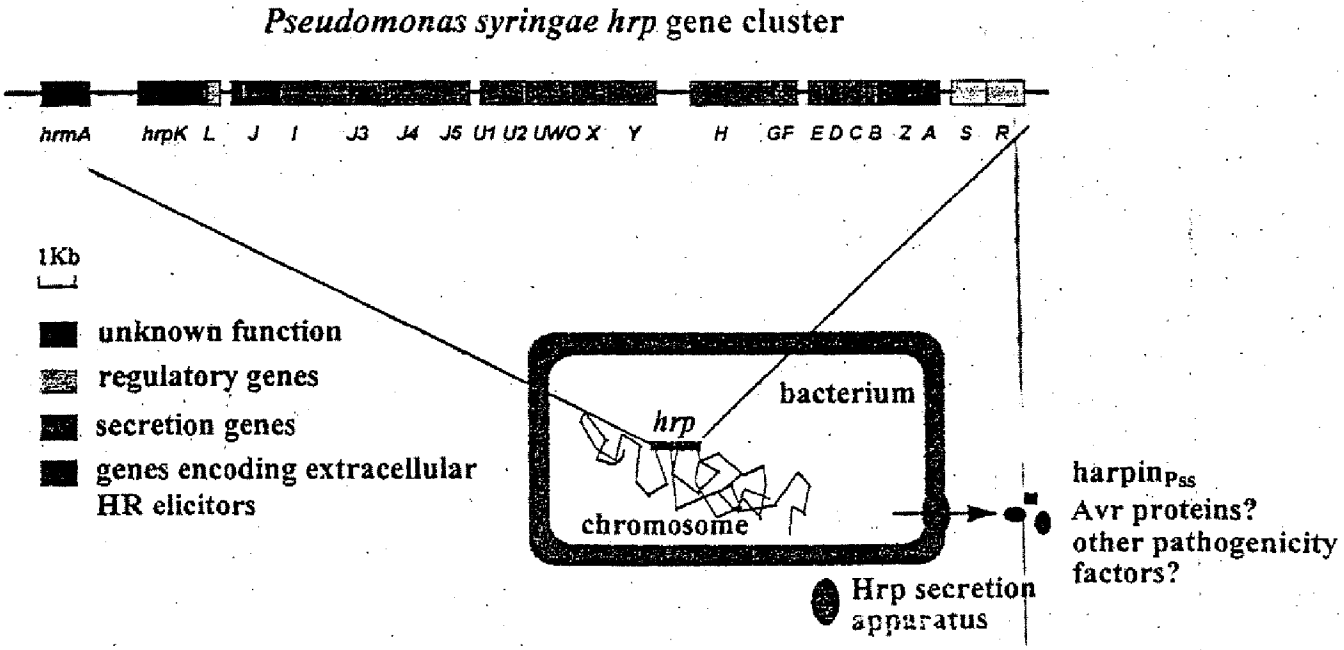

Fig. 2. *hrp* genes of *Pseudomonas syringae*. There are at least 25 *hrp* genes (*hrpA* to *hrpZ*) in *P. syringae*. *hrpS*, *hrpR*, and *hrpL* (in yellow) are involved in the detection of the plant apoplast environment and in the activation of all other *hrp* genes, *avr* genes, and possibly other pathogenicity-related genes. Most other *hrp* genes (in red) are involved in the assembly of the Hrp secretion apparatus in the bacterial envelope, through which travels a newly discovered class of bacterial virulence/avirulence proteins (in green), including HrpZ.

be dispensable for pathogenicity of *P. solanacearum* in the susceptible host plant, tomato, or for HR elicitation in the nonhost plant, tobacco (2), indicating that there must be other HR-elicitors and pathogenicity factors that traverse the Hrp secretion apparatus in this bacterium. Further examination indicated that PopA1 may function as an avirulence gene, mediating gene-for-gene interaction in certain Petunia–*P. solanacearum* interactions (2,45). Thus, the Hrp secretion apparatus in each bacterium may secrete a different number of proteins. Identification of other proteins that traverse the Hrp secretion apparatus is now an active research area and is essential for a complete understanding of *hrp*-mediated plant–bacterial interactions.

Factors Modifying *hrp* Gene-Mediated Compatibility

Two broad classes of bacterial genes may superimpose their functions on the *hrp* gene-mediated compatibility or incompatibility between plants and bacteria: *avr* genes and various virulence genes. The *avr* genes mediate genotype-specific incompatibility in resistant host plants. Virulence genes promote the production of disease symptoms and are usually needed for the full virulence of bacteria.

Bacterial *avr* Genes

Flor (14) formulated the gene-for-gene hypothesis in his work on flax–flax rust interactions. Flor hypothesized that the resistance of a given flax cultivar to a given fungal race is the result of the interaction between a fungal *avr* gene and a corresponding flax resistance gene. Therefore, the interactions between the plant's resistance genes and the pathogen's *avr* genes would limit the host range of the pathogen. Staskawicz et al. (44) first cloned an *avr* gene from a soybean bacterial pathogen, *Pseudomonas syringae* pv. *glycinea*, and showed that the cloned *avr* gene could convert virulent *P. s.* pv. *glycinea* strains that cause disease into avirulent strains that elicit the HR in certain soybean cultivars carrying the corresponding resistance genes, thus validating the role of *avr* genes in controlling host range. Since then, numerous *avr* genes have been cloned from plant-pathogenic bacteria (27). Several plant resistance genes have also been cloned using molecular genetic approaches (e.g., 34,43).

What is the relationship between the *avr* genes and *hrp* genes, both of which are involved in eliciting the HR? Several laboratories have observed that *avr* genes cannot trigger the genotype-specific HR in *hrp* mutants, i.e., *avr* genes depend on functional *hrp* genes for expressing their phenotype (25,26,28,38,40). There are several ways of explaining such dependence (Fig. 4). One possibility is that Avr proteins are dependent on the Hrp secretion apparatus for secretion. Alternatively, Avr function requires a prior plant response elicited by the *hrp*-controlled extracellular factors (such as harpins). A third possibility is that Avr proteins, with no HR-eliciting activity by themselves, cause the cultivar-specific HR by either covalently modifying harpins or modulating the expression of harpins in a plant resistance gene–dependent manner yet to be understood. Finally, it is also possible that Avr proteins are secreted directly into the plant cell with the help of harpins, assuming that receptors for Avr proteins are inside the plant cell. Studies are being carried out to resolve these possibilities.

Bacterial Virulence Factors

The genetic diversity of plant-pathogenic bacteria is reflected in their ability to cause diverse disease symptoms ranging from soft rot to tissue necrosis to "wildfire." These diverse disease symptoms are likely the result of the action of several, sometimes unique, virulence factors produced by a given bacterium in addition to *hrp*-controlled pathogenicity factors. For example, research from many laboratories has shown that toxin production plays an important role in the formation of chlorosis and necrosis (3,19,49). Extracellular polysaccharides may be involved in the formation of water-soaking lesions (11,13) and in the production of wilt symptoms by clogging the plant vascular system (9). Plant cell wall–degrading enzymes are responsible for tissue disintegration and the appearance of the soft-rot symptom (7). Plant hormones produced by plant-pathogenic bacteria are involved in the induction of tissue deformation (42).

Both *hrp* genes and bacterial virulence factors are necessary for disease symptom production, but what is the relationship between them? A logical relationship would be that *hrp*-controlled extracellular factors are involved in obtaining nutrients in early stages of pathogenesis, whereas other virulence factors drive the initial compatible stage into a fully compatible one, leading to the production of various disease symptoms. At least two lines of

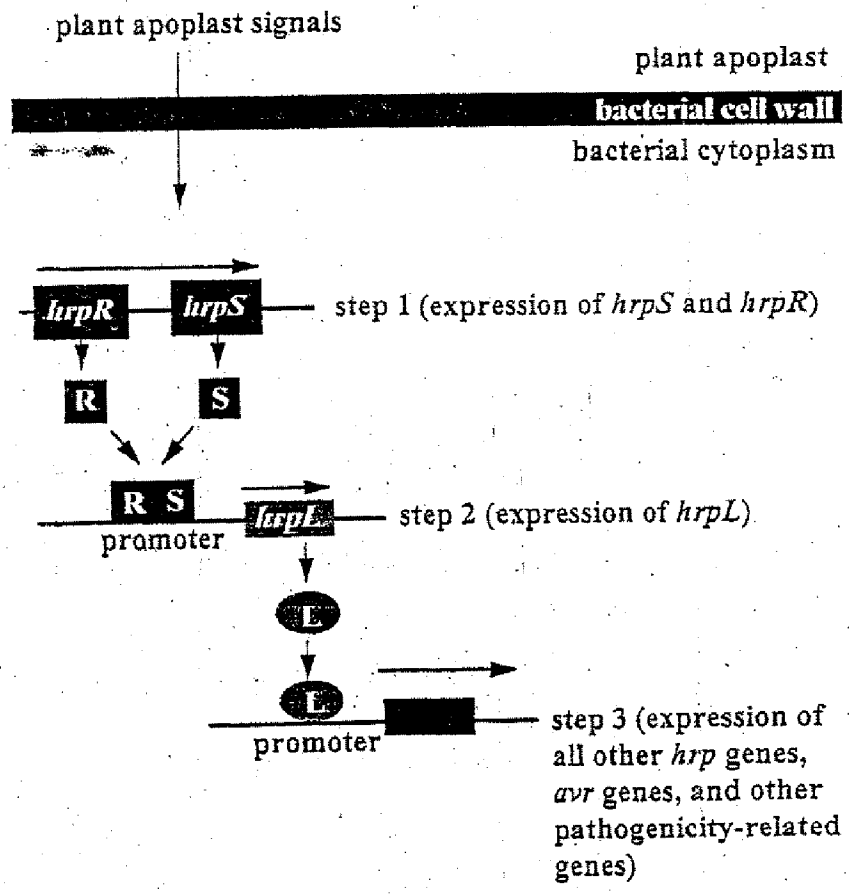

Fig. 3. Diagram of the signal transduction cascade in the detection of the plant apoplast environment by *Pseudomonas syringae*. The plant apoplast environment (limited nutrients and/or certain unique compounds) activates the expression of *hrpS* and *hrpR* by a mechanism yet to be understood (step 1). The *hrpS* and *hrpR* gene products (S and R, respectively) bind to and activate the promoter of the *hrpL* gene (step 2). The *hrpL* gene product (L), in turn, binds to promoters of other *hrp* genes, *avr* genes, and other bacterial pathogenicity-related genes to promote the expression of these genes, resulting in the initiation of diverse plant–bacteria interactions (step 3). Modified from Xiao et al. (51).

evidence seem to support this relationship. First, *hrp* genes are highly conserved among diverse plant-pathogenic bacteria, whereas virulence factors vary greatly among bacteria. Second, while mutations in the *hrp* gene completely abolish both bacterial pathogenicity and elicitation of the HR, mutations in virulence genes (e.g., toxin-production genes) often do not eliminate pathogenicity and have no effect on bacterial elicitation of the HR (3,19,49).

hrp Gene Functions and Disease Management

A major reason for discovering bacterial and plant factors critical for bacterial pathogenesis and plant resistance is to develop novel and environmentally safe strategies for controlling plant diseases. The discovery that the Hrp secretion apparatus is crucial to bacterial pathogenesis provides a foundation for designing novel chemicals and antibodies that would block the assembly of the Hrp secretion apparatus or the passage of bacterial virulence proteins through it. Alternatively, susceptible crop plants could be genetically engineered with genes encoding proteinaceous HR elicitors, such as harpins, under the control of plant promoters inducible by virulent pathogens. If this approach were successful, the HR or resistance would be triggered in otherwise compatible interactions, limiting disease development.

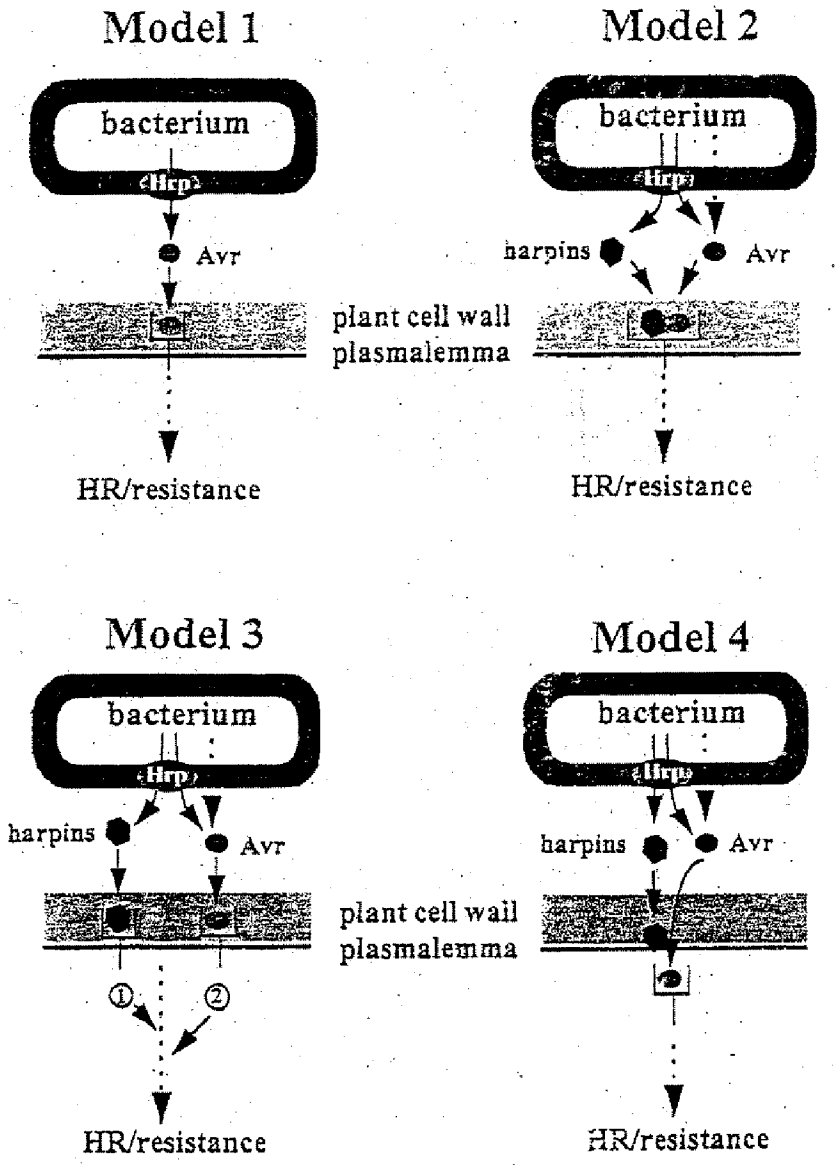

Fig. 4. Working models for possible interactions between *hrp* genes and *avr* genes. Model 1: Avr signals (Avr proteins or their enzymatic products) are secreted through the Hrp secretion apparatus to elicit the hypersensitive response (HR) and resistance. Model 2: Harpins and Avr signals modify each other before interacting with plant receptors. Avr signals may or may not be secreted via the Hrp secretion apparatus. Model 3: Harpins and Avr signals interact with respective plant receptors. Plant response elicited by harpins must precede plant response elicited by Avr signals. Avr signals may or may not be secreted via the Hrp secretion apparatus. Model 4: Avr proteins are secreted into the plant cell with the help of harpins. Avr signals may or may not be secreted via the Hrp secretion apparatus. In models 1 to 3, receptors for Avr proteins are presumed to be on the plant cell surface. In model 4, receptors for Avr proteins are inside the plant cell.

Acknowledgments

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Erwinia chrysanthemi Harpin$_{Ech}$: An Elicitor of the Hypersensitive Response that Contributes to Soft-Rot Pathogenesis

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Mutants of the soft-rot pathogen *Erwinia chrysanthemi* EC16 that are deficient in the production of the pectate lyase isozymes PelABCE can elicit the hypersensitive response (HR) in tobacco leaves. The *hrpN*$_{Ech}$ gene was identified in a collection of cosmids carrying *E. chrysanthemi hrp* genes by its hybridization with the *Erwinia amylovora hrpN*$_{Ea}$ gene. *hrpN*$_{Ech}$ appears to be in a monocistronic operon, and it encodes a predicted protein of 340 amino acids that is glycine-rich, lacking in cysteine, and highly similar to HrpN$_{Ea}$ in its C-terminal half. *Escherichia coli* DH5α cells expressing *hrpN*$_{Ech}$ from the *lac* promoter of pBluescript II accumulated HrpN$_{Ech}$ in inclusion bodies. The protein was readily purified from cell lysates carrying these inclusion bodies by solubilization in 4.5 M guanidine-HCl and reprecipitation upon dialysis against dilute buffer. HrpN$_{Ech}$ suspensions elicited a typical HR in tobacco leaves, and elicitor activity was heat-stable. Tn*5-gusA1* mutations were introduced into the cloned *hrpN*$_{Ech}$ and then marker-exchanged into the genomes of *E. chrysanthemi* strains AC4150 (wild type), CUCPB5006 (Δ*pelABCE*), and CUCPB5030 (Δ*pelABCE outD*::Tn*phoA*). *hrpN*$_{Ech}$::Tn*5-gusA1* mutations in CUCPB5006 abolished the ability of the bacterium to elicit the HR in tobacco leaves unless complemented with an *hrpN*$_{Ech}$ subclone. An *hrpN*$_{Ech}$::Tn*5-gusA1* mutation also reduced the ability of AC4150 to incite infections in witloof chicory leaves, but it did not reduce the size of lesions that did develop. Purified HrpN$_{Ech}$ and *E. chrysanthemi* strains CUCPB5006 and CUCPB5030 elicited HR-like necrosis in leaves of tomato, pepper, African violet, petunia, and pelargonium, whereas *hrpN*$_{Ech}$ mutants did not. HrpN$_{Ech}$ thus appears to be the only HR elicitor produced by *E. chrysanthemi* EC16, and it contributes to the pathogenicity of the bacterium in witloof chicory.

The hypersensitive response (HR) is a rapid, localized necrosis that is associated with the active defense of plants against many pathogens (Kiraly 1980; Klement 1982). The HR elicited by bacteria is readily observed as a tissue collapse if high concentrations (≥10^7 cells per milliliter) of a limited-host-range pathogen like *Pseudomonas syringae* or *Erwinia amylovora* are infiltrated into leaves of nonhost plants (necrosis occurs only in isolated plant cells at lower levels of inoculum) (Klement 1963; Klement *et al.* 1964; Turner and Novacky 1974; Klement 1982). The capacities to elicit the HR in a nonhost and to be pathogenic in a host appear linked. As noted by Klement (1982), these pathogens also cause physiologically similar, albeit delayed, necroses in their interactions with compatible hosts. Furthermore, the ability to produce the HR or pathogenesis is dependent on a common set of genes, denoted *hrp* (Lindgren *et al.* 1986; Willis *et al.* 1991). Consequently, the HR may hold clues to both the nature of plant defense and the basis for bacterial pathogenicity.

The *hrp* genes are widespread in gram-negative plant pathogens, where they are clustered, conserved, and in some cases interchangeable (Willis *et al.* 1991; Bonas 1994). Several *hrp* genes encode components of a protein secretion pathway similar to one used by *Yersinia*, *Shigella*, and *Salmonella* spp. to secrete proteins essential in animal diseases (Van Gijsegem *et al.* 1993). In *E. amylovora*, *P. syringae*, and *P. solanacearum*, *hrp* genes have been shown to control the production and secretion of glycine-rich protein elicitors of the HR (He *et al.* 1993; Wei and Beer 1993; Arlat *et al.* 1994).

The first of these proteins was discovered in *E. amylovora* Ea321, a bacterium that causes fire blight of rosaceous plants, and was designated harpin (Wei *et al.* 1992). Mutations in the encoding *hrpN* gene revealed that harpin is required for *E. amylovora* to elicit the HR in nonhost tobacco leaves and incite disease symptoms in highly susceptible pear fruit. The *P. solanacearum* GMI1000 PopA1 protein has similar physical properties and also elicits the HR in leaves of tobacco, which is not a host of that strain (Arlat *et al.* 1994). However, *P. solanacearum popA* mutants still elicit the HR in tobacco and incite disease in tomato. Thus, the role of these glycine-rich HR elicitors can vary widely among gram-negative plant pathogens.

E. chrysanthemi is unlike the bacterial pathogens that typically elicit the HR because it has a wide host range, rapidly kills and macerates host tissues, and secretes several isozymes of the macerating enzyme pectate lyase (Pel) (Barras *et al.* 1994). Nevertheless, PelABCE$^-$ and Out$^-$ (pectic enzyme secretion pathway) mutants of *E. chrysanthemi* EC16 cause a typical HR (Bauer *et al.* 1994). Furthermore, elicitation of the HR by *E. chrysanthemi* is dependent on an *hrp* gene that is conserved in *E. amylovora* and *P. syringae* and functions in the secretion of the *E. amylovora* harpin (Wei and Beer 1993; Bauer *et al.* 1994). Mutation of this gene significantly reduces the ability of *E. chrysanthemi* to incite lesions in susceptible

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witloof chicory leaves. These observations suggest that *E. chrysanthemi* also produces a harpin. We report here the cloning, characterization, and mutagenesis of the *E. chrysanthemi* $hrpN_{Ech}$ gene and an investigation of the role of its product in plant interactions.

RESULTS

Molecular cloning of the *E. chrysanthemi* $hrpN_{Ech}$ gene.

We previously isolated 18 cosmids containing *E. chrysanthemi* DNA sequences hybridizing with the *E. amylovora hrp* cluster (Bauer *et al.* 1994). The pattern of restriction fragments released from these cosmids indicated they all contained overlapping inserts from the same region of the *E. chrysanthemi* genome (data not shown). The cosmids were probed in colony blots with a 1.3-kb *Hin*dIII fragment from pCPP1084, which contains the *E. amylovora hrpN* gene (Wei *et al.* 1992). pCPP2157, one of the three cosmids hybridizing with the probe, was digested with several restriction enzymes, and the location of the $hrpN_{Ech}$ gene in those fragments was determined by probing a Southern blot with the *E. amylovora* *Hin*dIII fragment. Two fragments, each containing the entire $hrpN_{Ech}$ gene, were subcloned into different vectors: pCPP2142 contained an 8.3-kb *Sal*I fragment in pUC119 (Vieira and Messing 1987), and pCPP2141 contained a 3.1-kb *Pst*I fragment in pBluescript II SK(–) (Stratagene, La Jolla, CA).

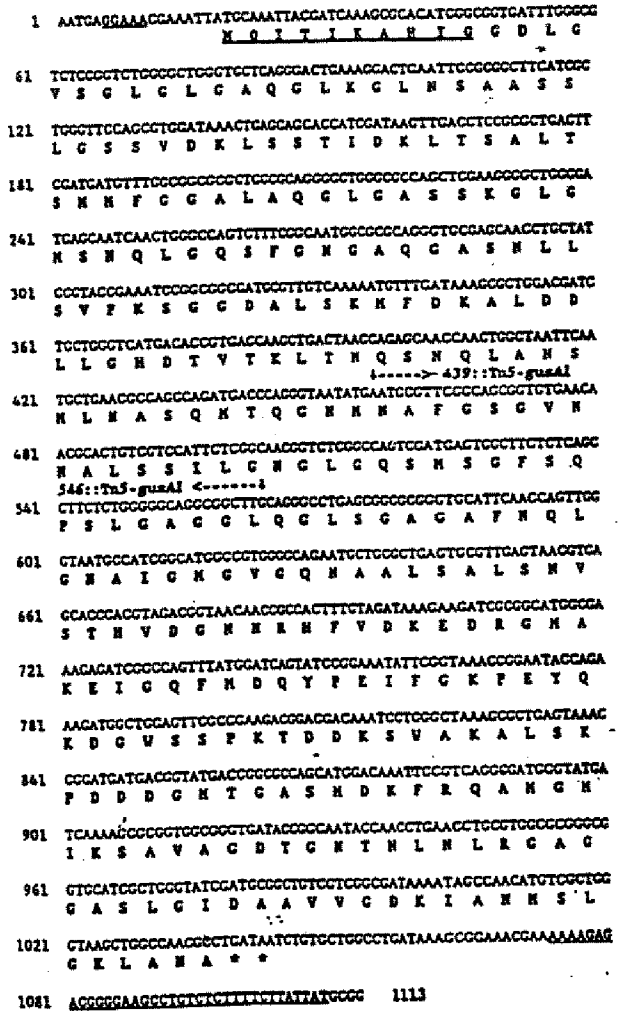

Fig. 1. DNA sequence of $hrpN_{Ech}$ and predicted amino acid sequence of its product. Underlined are the putative ribosome-binding site, the N-terminal amino acids confirmed by sequencing the product of pCPP2172, and a potential rho-independent transcription terminator. The location and orientation of two Tn*5-gusAI* insertions are also indicated and are numbered according to their location in the $hrpN_{Ech}$ open reading frame. The accession number for *hrpN* is L39897.

Sequence of $hrpN_{Ech}$.

The nucleotide sequence of a 2.4-kb region of pCPP2141 encompassing $hrpN_{Ech}$ was determined. The portion of that sequence extending from the putative ribosome-binding site through the $hrpN_{Ech}$ coding sequence to a putative rho-independent terminator is presented in Figure 1. The typical ribosome-binding site, consisting of GGAAA, was located eight bases upstream of the ATG translational initiation codon. A consensus *hrp* promoter sequence of $GGAACC(N)_{16}CACTCA$ (Bonas 1994) was found 97 bases upstream of the open reading frame (ORF), suggesting that $hrpN_{Ech}$ is a monocistronic operon. $hrpN_{Ech}$ codes for a predicted protein that has a molecular mass of 34.3 kDa, is rich in glycine (16.2%), and is lacking in cysteine. Comparison of the amino acid sequences of the predicted $hrpN_{Ea}$ and $hrpN_{Ech}$ products revealed extensive similarity, particularly in the C-terminal halves of the proteins (Fig. 2). The overall identity of the

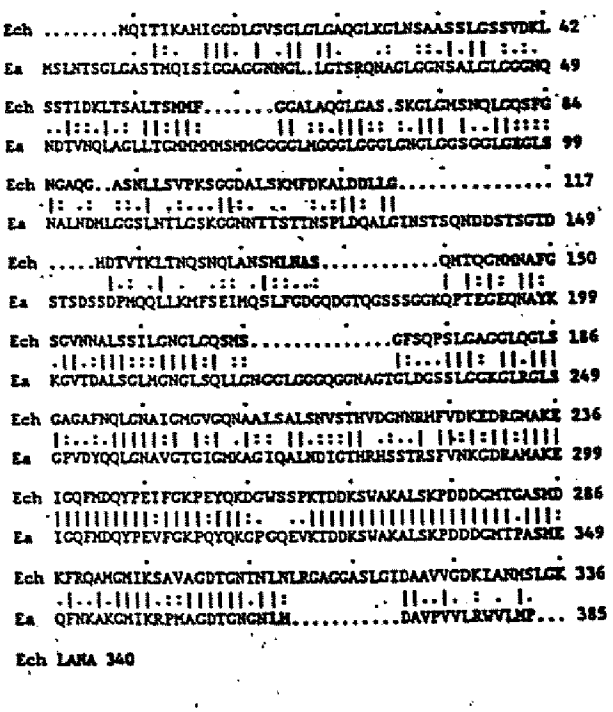

Fig. 2. Predicted amino acid sequences of the *hrpN* products $HrpN_{Ech}$ (Ech) of *Erwinia chrysanthemi* and $HrpN_{Ea}$ (Ea) of *E. amylovora*, aligned by the Gap program of the Genetics Computer Group Sequence Analysis Software Package (Devereaux *et al.* 1984). Two dots denote greater similarity than one dot.

hrpN genes and proteins was 66.9 and 45.5%, as determined by the FASTA and Gap algorithms, respectively (Devereaux *et al.* 1984; Pearson and Lipman 1988).

The direction of $hrpN_{Ech}$ transcription, the size of the predicted product, and the translation start site were confirmed by recloning the 3.1-kb *Pst*I fragment from pCPP2157 and selecting a clone with the fragment in pBluescript II SK(–) in the opposite orientation from pCPP2141, to produce pCPP2172. *E. coli* DH5α(pCPP2172) expressed $hrpN_{Ech}$ from the vector *lac* promoter and produced high levels of a protein with an estimated molecular mass of 36 kDa in sodium dodecyl sulfate (SDS) polyacrylamide gels, which is close to the predicted size (Fig. 3). Furthermore, the 10 N-terminal amino acids of the 36-kDa protein, determined by microsequencing following purification as described below, corresponded with the predicted N terminus of $HrpN_{Ech}$. As expected, no N-terminal signal sequence for targeting to the general export (Sec) pathway was discernible in the $HrpN_{Ech}$ sequence, and our data showed no evidence of processing of the N terminus.

Purification of the $hrpN_{Ech}$ product and demonstration of its HR elicitor activity in tobacco.

When DH5α(pCPP2172) cells were disrupted by sonication and then centrifuged, most of the $HrpN_{Ech}$ protein sedimented with the cell debris. However, soluble $HrpN_{Ech}$ could be released from this material by treatment with 4.5 M guanidine-HCl. This suggested that the protein formed inclusion bodies which could be exploited for purification. As detailed in Materials and Methods, we found that $HrpN_{Ech}$ reprecipitated when the guanidine-HCl was removed by dialysis against dilute buffer. The $HrpN_{Ech}$ precipitate could be washed and resuspended in buffer, in which it formed a fine suspension. SDS polyacrylamide gel analysis revealed the suspension to be electrophoretically homogeneous $HrpN_{Ech}$ (Fig. 3).

Cell-free lysates from *E. coli* DH5α(pCPP2172) cells grown in Luria-Bertani medium were infiltrated into tobacco leaves. Necrosis typical of the HR developed within 18 h, whereas leaf panels infiltrated with identically prepared lysates of DH5α(pBluescript SK–) showed no response (data not shown). The suspension of purified $HrpN_{Ech}$ at a concentration of 336 μg/ml also caused a necrotic response within 18 h that was indistinguishable from that caused by *E. chrysanthemi* CUCBP5030 or cell-free lysates from *E. coli* DH5α(pCPP2172) (Fig. 4). Tobacco plants vary in their sensitivity to harpins, and elicitation of the HR by $HrpN_{Ech}$ at lower concentrations was found to be variable. Consequently, a concentration of 336 μg/ml was used in all subsequent experiments. The concentration of $HrpN_{Ech}$ that is soluble in apoplastic fluids is unknown. To determine the heat stability of $HrpN_{Ech}$, the suspension of purified protein was incubated at 100° C for 15 min and then infiltrated into a tobacco leaf. There was no apparent diminution in its ability to elicit the HR (data not shown). These observations indicated that $HrpN_{Ech}$ is sufficient to account for the ability of *E. chrysanthemi* to elicit the HR in tobacco.

$hrpN_{Ech}$ mutants fail to elicit the HR in tobacco.

E. coli DH10B(pCPP2142) was mutagenized with Tn*5-gusA1* (Sharma and Signer 1990). Plasmid DNA was isolated

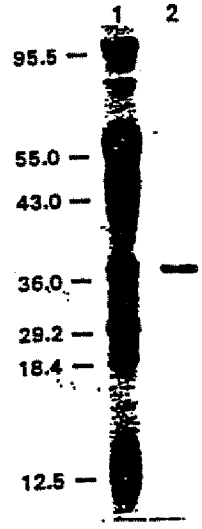

Fig. 3. Sodium dodecyl sulfate (SDS) polyacrylamide gel of purified $HrpN_{Ech}$. Purified $HrpN_{Ech}$ was solubilized in SDS loading buffer, electrophoresed through a 12% polyacrylamide gel, and stained with Coomassie Brilliant Blue. Lane 1, molecular weight markers (mid-range markers from Diversified Biotech, Boston, MA), with sizes in kilodaltons shown to the left; lane 2, $HrpN_{Ech}$.

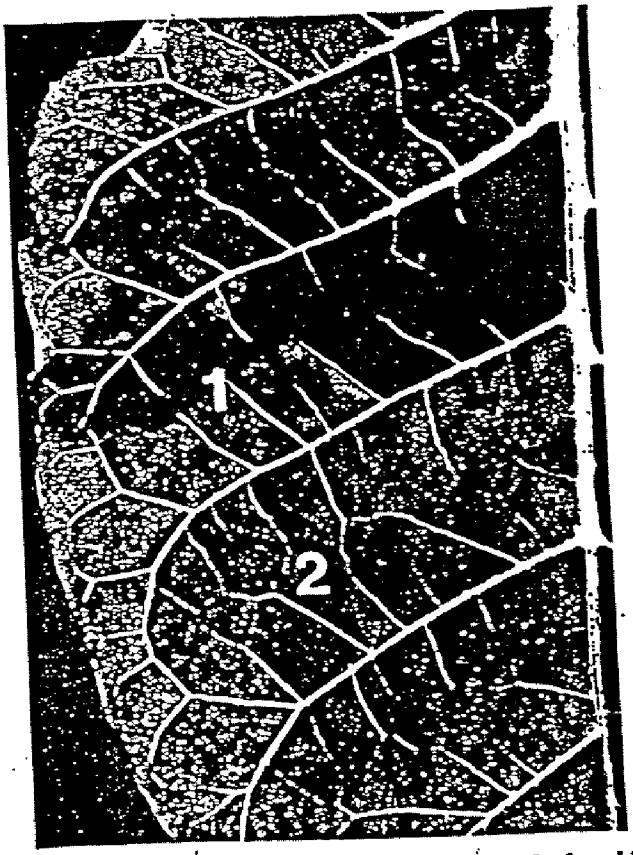

Fig. 4. Response of tobacco leaf tissue to purified $HrpN_{Ech}$. Leaf panel 1 was infiltrated with a suspension of purified $HrpN_{Ech}$ at a concentration of 336 μg/ml in 5 mM morpholinoethanesulfonic acid, pH 6.5. Panel 2 was infiltrated with buffer alone. The tissue in panel 1 collapsed 18 hr later. The leaf was photographed, 24 hr after infiltration, with a cross-polarized transilluminator, which enhances black and white visualization by making necrotic, desiccated areas that are typical of the hypersensitive response appear black.

from kanamycin-resistant colonies and transformed into *E. coli* DH5α, with selection for kanamycin resistance. Plasmids containing Tn*5-gusA1* were analyzed by restriction mapping. Two independent insertions in an 0.82-kb *Cla*I fragment internal to $hrpN_{Ech}$ were chosen for further study. The precise location and orientation of these insertions was determined by using a primer that hybridizes to Tn*5-gusA1* DNA upstream of *gusA* to sequence into the disrupted *E. chrysanthemi* DNA (Fig. 1). *E. coli* DH5α(pCPP2142) cells carrying the Tn*5-gusA1* insertion at nucleotide 439 of the $hrpN_{Ech}$ ORF (with *gusA* and $hrpN_{Ech}$ in the same orientation) produced dark blue colonies indicative of β-glucuronidase activity on LM agar (Hanahan 1983) supplemented with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (data not shown). Whether *gusA* was expressed from an *E. chrysanthemi* promoter or the vector *lac* promoter was not determined. The $hrpN_{Ech}439$::Tn*5-gusA1* and $hrpN_{Ech}546$::Tn*5-gusA1* mutations were marker-exchanged into the genome of *E. chrysanthemi* CUCPB5006 (Δ*pelABCE*) to produce mutants CUCPB5046 and CUCPB5045, respectively. Neither of the $hrpN_{Ech}$ mutants elicited a visible reaction in tobacco leaves (Fig. 5).

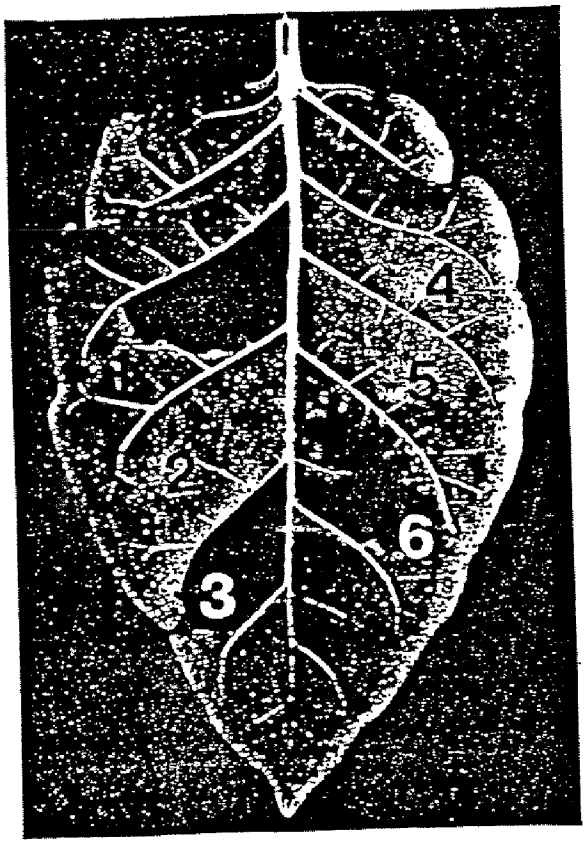

Fig. 5. Tobacco leaf showing that *Erwinia chrysanthemi hrpN* mutants do not elicit the hypersensitive response unless complemented with $hrpN^+$ pCPP2174. Bacteria were suspended at a concentration of 5×10^8 cells per milliliter in 5 mM morpholinoethanesulfonic acid, pH 6.5, and infiltrated into a tobacco leaf. The leaf was photographed 24 hr later under cross-polarized transillumination, as in Figure 4. 1, *E. chrysanthemi* CUCPB5006 (Δ*pelABCE*); 2, CUCPB5045 (Δ*pelABCE* $hrpN_{Ech}546$::Tn*5-gusA1* derivative of CUCPB5006); 3, CUCPB5045(pCPP2174); 4, buffer alone; 5, CUCPB5046 (Δ*pelABCE* $hrpN_{Ech}439$::Tn*5-gusA1* derivative of CUCPB5006); 6, CUCPB5046(pCPP2174).

E. chrysanthemi $hrpN_{Ech}$ mutations can be complemented *in trans* with $hrpN_{Ech}$ but not with $hrpN_{Ea}$.

The presence of a typical rho-independent terminator just downstream of the $hrpN_{Ech}$ ORF suggested that mutations in the gene would not have polar effects on any other genes and that the HR elicitation phenotype should be restored by an $hrpN_{Ech}$ subclone. Because pCPP2172 carried 2 kb of *E. chrysanthemi* DNA in addition to $hrpN_{Ech}$, we constructed a precise subclone of the gene for this purpose. Oligonucleotides were used to amplify the $hrpN_{Ech}$ ORF by polymerase chain reaction and to introduce terminal *Nco*I and *Xho*I sites. The introduction of the restriction sites resulted in changing the second residue in the protein from glutamine to valine and adding a leucine and a glutamic acid residue to the C terminus. The resulting DNA fragment was ligated into *Xho*I- and *Nco*I-digested pSE280, creating pCPP2174, in which $hrpN_{Ech}$ was under control of the vector *tac* promoter. *E. chrysanthemi* CUCPB5045(pCPP2174) and CUCPB5046(pCPP2174) possessed HR elicitor activity (Fig. 5). HR elicitor activity could also be restored to these mutants by pCPP2142 and pCPP2172, but not by pCPP2141 (data not shown). Thus, the production of $HrpN_{Ech}$ is essential for elicitation of the HR by *E. chrysanthemi* CUCPB5006.

The feasibility of testing the interchangeability of the *hrpN* genes of *E. chrysanthemi* and *E. amylovora* was supported by the observation that HR elicitation activity could be restored to *hrpN* mutants in each species (*E. chrysanthemi* CUCPB5045 and *E. amylovora* Ea321T5) by their respective $hrpN^+$ subclones (pCPP2142 and pCPP1084). pCPP2142 was used for this purpose because preliminary immunoblot experiments indicated that the level of $hrpN_{Ech}$ expression by this plasmid, though relatively high, most closely approximated the expression of the native *hrpN* gene in *E. amylovora*. However, despite good heterologous expression of the *hrpN* genes, HR elicitation activity was not restored in either *E. amylovora* Ea321T5(pCPP2142) or *E. chrysanthemi*(pCPP1084) (data not shown). Thus, the genes do not appear to be functionally interchangeable.

E. chrysanthemi $hrpN_{Ech}$ mutants have a reduced ability to incite lesions in witloof chicory.

The $hrpN_{Ech}439$::Tn*5-gusA1* mutation was marker-exchanged into the genome of wild-type strain AC4150. The resulting mutant, CUCPB5049, was analyzed for its virulence in witloof chicory. Leaves were inoculated at small wounds with

Table 1. Effects of $hrpN_{Ech}$ mutation on the ability of *Erwinia chrysanthemi* to incite lesions on witloof chicory leaves

Strain	Number of lesions per 20 inoculations[a]	Size of lesions (mm^2, mean ± SD)[b]
AC4150 (wild type)	16	80 ± 55
CUCPB5049 ($hrpN_{Ech}429$::Tn*5-gusA1*)	8[c]	89 ± 42

[a] Each witloof chicory leaf was inoculated at two equivalent sites with 2×10^4 bacterial cells: one site received the $hrpN_{Ech}$ mutant, the other the parental wild-type strain; lesions were indicated by browning and maceration around the site of inoculation.

[b] Product of the length and width of the lesion.

[c] Different from the wild-type strain ($P < 0.05$), as determined by the McNemar test (Conover 1980).

2×10^4 cells of mutant and wild-type strains, as previously described (Bauer *et al.* 1994). The level of inoculum corresponded with the experimentally determined ED_{50} of the wild-type strain for the batch of chicory heads used. The approximate surface area of macerated lesions was determined

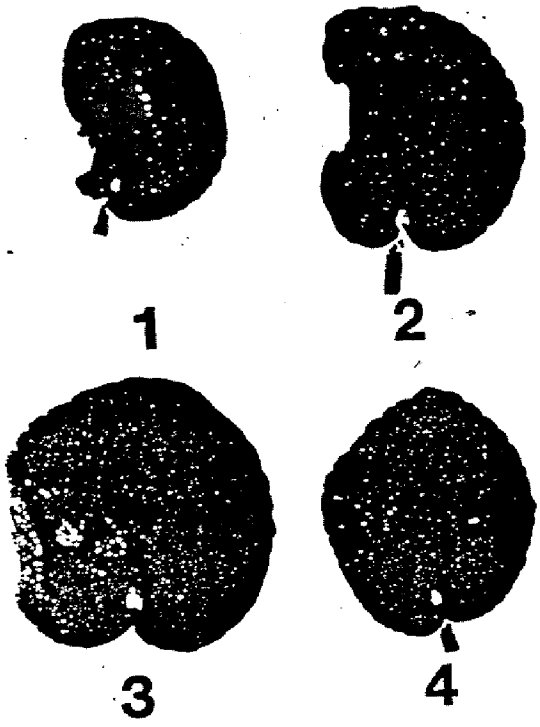

Fig. 6. African violet leaves showing rapid necrosis elicited by $HrpN_{Ech}$ and $HrpN^{-}_{Ech}$ Pel-deficient strains of *Erwinia chrysanthemi*. Leaves were inoculated with bacteria at a concentration of 3×10^8 cells per milliliter in 5 mM morpholinoethanesulfonic acid, pH 6.5, or purified $HrpN_{Ech}$ at a concentration of 336 μg/ml and photographed 24 hr later under cross-polarized transillumination, as in Figure 4. Buffer controls elicited no visible response (not shown). 1, *E. chrysanthemi* CUCPB5006 (Δ*pelABCE*); 2, CUCPB5030 (*outD*::Tn*phoA* derivative of CUCPB-5006); 3, $HrpN_{Ech}$; 4, (left) CUCPB5045 (Δ*pelABCE* $hrpN_{Ech}546$::Tn*5-gusA1* derivative of CUCPB5006) and (right) CUCPB5063 (Δ*pelABCE outD*::Tn*phoA* $hrpN_{Ech}546$::Tn*5-gusA1* derivative of CUCPB5006).

72 h after inoculation. The mutations did not abolish the pathogenicity of *E. chrysanthemi*, but they significantly reduced the number of successful lesions (Table 1). However, the $hrpN_{Ech}$ mutation had no significant effect on the size of the lesions produced in successful infections.

Elicitation of a rapid necrosis in several plants by *E. chrysanthemi* is dependent on $HrpN_{Ech}$.

To determine whether *E. chrysanthemi* could cause an $HrpN_{Ech}$-dependent necrosis in plants other than tobacco, a variety of plants were infiltrated with purified $HrpN_{Ech}$ or inoculated with Pel-deficient *E. chrysanthemi* strains. The strains used were CUCPB5006; its $hrpN_{Ech}546$::Tn*5-gusA1* derivative, CUCPB5045; CUCPB5030 (Δ*pelABCE outD*::Tn*phoA*); and its $hrpN_{Ech}546$::Tn*5-gusA1* derivative, CUCPB-5063. The results for African violet are shown in Figure 6, and results for all plants are summarized in Table 2. They yield several general observations. Plants responded either to both isolated $HrpN_{Ech}$ and $hrpN^{-}_{Ech}$ bacteria or to neither. Plants that responded to either treatment produced a non-macerated, HR-like necrosis that developed between 12 and 24 h after infiltration. $hrpN_{Ech}$ mutants failed to elicit a response in any of the plants. The *out* mutation had no apparent influence on the responses elicited in the plants tested, indicating that residual Pel isozymes or other proteins traveling the Out pathway were not involved in producing the HR-like necrosis. The results argue that $HrpN_{Ech}$ is the only elicitor of the HR produced by *E. chrysanthemi*.

DISCUSSION

E. chrysanthemi was found to produce a protein with many similarities to the harpin of *E. amylovora*. The two proteins share significant amino acid sequence identity, similar physical properties, and the ability to elicit the HR in a variety of plants. Mutations in the $hrpN_{Ech}$ gene indicate that, as with *E. amylovora*, harpin production is required for elicitation of the HR. Furthermore, both harpins contribute to bacterial pathogenicity, albeit to different degrees. $HrpN_{Ech}$ is essential for *E. amylovora* to produce symptoms in highly susceptible, immature pear fruit (Wei *et al.* 1992), whereas $HrpN_{Ech}$ merely increases the frequency of successful *E. chrysanthemi* infections in susceptible witloof chicory leaves. Nevertheless, the finding that harpins play some role in the pathogenicity of

Table 2. Elicitation of necrosis in various plants by $HrpN_{Ech}$ and by *Erwinia chrysanthemi* strains variously deficient in Pel production and $HrpN_{Ech}$ production

Plant	$HrpN_{Ech}$[a]	CUCPB5006 (Δ*pelABCE*)[b]	CUCPB5045 (Δ*pelABCE* $hrpN_{Ech}546$::Tn*5-gusA1*)	CUCPB5030 (Δ*pelABCE outD*::Tn*phoA*)	CUCPB5063 (Δ*pelABCE outD*::Tn*phoA* $hrpN_{Ech}546$::Tn*5-gusA1*)
Tobacco	+	+	−	+	−
Tomato	+	+	−	+	−
Pepper	+	+	−	+	−
African violet	+	+	−	+	−
Petunia	+	+	−	+	−
Pelargonium	+	+	−	+	−
Squash	−	−	−	−	−
Zinnia	−	−	−	−	−

[a] Leaves on plants were infiltrated with $HrpN_{Ech}$ at a concentration of 336 μg/ml and observed macroscopically 24 hr later for necrosis and collapse of the infiltrated area (+) or absence of any response (−).

[b] Leaves on plants were infiltrated with bacteria at a concentration of 5×10^8/ml and scored for responses as described above.

such disparate pathogens suggests that these proteins have a conserved and widespread function in bacterial plant pathogenesis. We will consider below $HrpN_{Ech}$ with regard to the protein secretion pathways, extracellular virulence proteins, and wide host range of *E. chrysanthemi*.

E. chrysanthemi secretes proteins by multiple, independent pathways. Several protease isozymes are secreted by the Sec-independent (ABC-transporter, or Type I) pathway; pectic enzymes and cellulase are secreted by the Sec-dependent (general secretion, or Type II) pathway; and, $HrpN_{Ech}$ is likely to be secreted by the Sec-independent Hrp (Type III) pathway (Salmond 1994). The expectation that $HrpN_{Ech}$ is secreted by the Hrp pathway is supported by several lines of indirect evidence: (i) Hrp secretion pathway mutants have revealed that other members of this class of glycine-rich, heat-stable elicitor proteins—the *E. amylovora* $HrpN_{Ea}$, *P. syringae* pv. *syringae* HrpZ, and *P. solanacearum* PopA1 proteins—are secreted by this pathway (He *et al.* 1993; Wei and Beer 1993; Arlat *et al.* 1994); (ii) mutation of the *E. chrysanthemi* homolog of an *E. amylovora* gene involved in $HrpN_{Ea}$ secretion abolishes the ability of *E. chrysanthemi* to elicit the HR, whereas mutation of the Out (Type II) pathway of *E. chrysanthemi* does not abolish the HR; and (iii) $HrpN_{Ech}$ appears to be the only HR elicitor produced by *E. chrysanthemi* (as discussed further below), suggesting that the effect of the putative *hrp* secretion gene mutation is on $HrpN_{Ech}$. Our attempts to directly demonstrate *hrp*-dependent secretion of $HrpN_{Ech}$ have been thwarted by the apparent instability of the protein in *E. chrysanthemi*. Using the cell fractionation and immunoblotting procedures of He *et al.* (1993) and polyclonal anti-$HrpN_{Ea}$ antibodies that cross-react with $HrpN_{Ech}$ (Wei *et al.* 1992), we have observed the presence of $HrpN_{Ech}$ in the cell-bound fraction of *E. chrysanthemi* (D. W. Bauer, unpublished). However, some culture preparations unexpectedly lack the protein, and no preparations reveal accumulation of the protein in the culture supernatant fraction. It is possible that $HrpN_{Ech}$ aggregates upon secretion and therefore precipitates from the medium. It is interesting that several of the *Yersinia* spp. Yop virulence proteins aggregate in the medium upon secretion via the Type III pathway (Michiels *et al.* 1990). Similarly, $HrpN_{Ea}$ has a propensity to form aggregates or to associate with an insoluble membrane fraction (Wei *et al.* 1992).

It is significant that there is little difference in the plant interaction phenotypes of *E. chrysanthemi* mutants deficient in either $HrpN_{Ech}$ or a putative component of the Hrp secretion pathway (Bauer *et al.* 1994). Both mutations abolish the ability of Pel-deficient strains to elicit the HR, and they both reduce the frequency of successful infections incited by fully pectolytic strains in witloof chicory leaves without affecting

Table 3. Bacterial strains and plasmids used in this study

Designation	Relevant characteristic[a]	Reference or source
Escherichia coli		
ED8767	*supE*44 *supF*58 *hsdS*3($r_B^-m_B^-$) *recA*56 *galk*2 *galT*22 *metB*1	Sambrook *et al.* 1989
DH5α	*supE*44 Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1 Nal^r	Hanahan 1983 Life Technologies, Inc., Grand Island, NY
DH10B	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80 *lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD*139 Δ(*ara, leu*)7697 *galU galK rpsL nupG*	Grant *et al.* 1990 Life Technologies, Inc.
Erwinia chrysanthemi		
EC16	Wild-type strain	Burkholder *et al.* 1953
AC4150	Spontaneous Nal^r derivative of EC16	Chatterjee *et al.* 1983
CUCPB5006	Δ(*pelB pelC*)::28bp Δ(*pelA pelE*) derivative of AC4150	He and Collmer 1990
CUCPB5030	*outD*::Tn*phoA* derivative of CUCPB5006	Bauer *et al.* 1994
CUCPB5045	$hrpN_{Ech}$*546*::Tn*5-gusA1* derivative of CUCPB5006	This work
CUCPB5046	$hrpN_{Ech}$*439*::Tn*5-gusA1* derivative of CUCPB5006	This work
CUCPB5063	$hrpN_{Ech}$*546*::Tn*5-gusA1* derivative of CUCPB5030	This work
CUCPB5049	$hrpN_{Ech}$*439*::Tn*5-gusA1* derivative of AC4150	This work
Erwinia amylovora		
Ea321	Wild type	ATCC 49947
Ea321T5	$hrpN_{Ea}$::Tn*5tac1* derivative of Ea321	Wei *et al.* 1992
Plasmids and phage		
pBluescript II SK(−)	Amp^r	Stratagene, La Jolla, CA
pCPP19	Cosmid vector, Sp^r/Sm^r	D. W. Bauer
pUC119	Amp^r plasmid vector	Vieira and Messing 1987
pSE280	Amp^r plasmid vector with superpolylinker downstream of tac promoter	Brosius 1989
pCPP2030	pCPP19 carrying *E. chrysanthemi* DNA hybridizing with *E. amylovora hrp* genes in pCPP1033	Bauer *et al.* 1994
pCPP1084	pBluescript M13+ carrying $hrpN_{Ech}$ on 1.3-kb *Hin*dIII fragment	Wei *et al.* 1992
pCPP2157	pCPP19 carrying *E. chrysanthemi* DNA hybridizing with *E. amylovora hrpN*	This work
pCPP2142	8.3-kb *Sal*I subclone from pCPP2157 in pUC119	This work
pCPP2141	3.1-kb *Pst*I subclone from pCPP2157 in pBluescript II SK(−) $hrpN_{Ech}$ in the orientation opposite that of the vector *lac* promoter	This work
pCPP2172	3.1-kb *Pst*I subclone from pCPP2157 in pBluescript II SK(−) $hrpN_{Ech}$ in same orientation as vector *lac* promoter	This work
pCPP2174	1.0-kb $hrpN_{Ech}$ polymerase chain reaction product cloned in *Nco*I-*Hin*dIII sites of pSE280	This work
λ::Tn*5-gusA1*	Tn*5* derivative for generating transcriptional fusions with *uidA* reporter; Kan^r, Tet^r	Sharma and Signer 1990

[a] Amp^r = ampicillin resistance; Kan^r = kanamycin resistance; Nal^r = nalidixic acid resistance; Sm^r = streptomycin resistance; Sp^r = spectinomycin resistance; Tet^r = tetracycline resistance.

the size of the macerated lesions that do develop. This pattern contrasts with that observed in mutations affecting Pel isozymes and the Out pathway. Virulence, as measured by maceration, is merely reduced by individual *pel* mutations, whereas it is abolished by *out* mutations. This is because multiple Pel isozymes (and possibly other enzymes) contribute quantitatively to virulence, but all of the Pel isozymes appear to be dependent on the Out pathway for secretion from the bacterial cell. The simplest interpretation of the observations with *E. chrysanthemi hrp* mutants is that $HrpN_{Ech}$ is the only protein traveling the Hrp pathway that has a detectable effect on the interaction of *E. chrysanthemi* EC16 with the plants tested.

The primacy of $HrpN_{Ech}$ in the *E. chrysanthemi* Hrp system is further supported by the observations that $hrpN_{Ech}$ mutants failed to elicit necrosis in any of the several plants tested and that all plants responding with apparent hypersensitivity to $HrpN^{+}_{Ech}$ strains also responded to isolated $HrpN_{Ech}$. Several of the plants sensitive to $HrpN_{Ech}$ are also susceptible to bacterial soft rots. This is particularly significant for African violet, whose interactions with *E. chrysanthemi* have been extensively studied (Barras *et al.* 1994). Thus, $HrpN_{Ech}$ elicits HR-like responses in plants that are susceptible to *E. chrysanthemi* infections under appropriate environmental conditions. The significance of this for the wide host range of the bacterium requires further investigation, and virulence tests with $hrpN_{Ech}$ mutants and additional susceptible plants are needed to determine the general importance of $HrpN_{Ech}$ and the Hrp system in *E. chrysanthemi*. For example, our present data do not address the possibility that other proteins secreted by the Hrp pathway, which are not elicitors of the HR in the plants we tested, may contribute to pathogenesis in hosts other than witloof chicory.

An important question is whether bacteria expressing heterologous harpins will be altered in pathogenicity. The *hrpN* genes of *E. chrysanthemi* and *E. amylovora* are particularly attractive for experiments addressing this because of the similarity of the harpins and the dissimilarity of the diseases produced by these bacteria. Unfortunately, attempts to restore the HR phenotype to *E. chrysanthemi* and *E. amylovora hrpN* mutants with heterologous $hrpN^{+}$ subclones failed. Since the *hrpN* genes in each subclone successfully complemented *hrpN* mutations in homologous bacteria and were expressed in heterologous bacteria, the problem is most likely the secretion of the harpins by heterologous Hrp systems. A similar problem has been encountered with heterologous secretion of Pel and cellulase via the Out pathway in *E. chrysanthemi* and *E. carotovora*, species that are more closely related to each other in this rather heterogeneous genus than *E. chrysanthemi* and *E. amylovora* are (He *et al.* 1991; Py *et al.* 1991).

In conclusion, two classes of proteins contribute to the pathogenicity of *E. chrysanthemi*—a single harpin and a battery of plant cell wall–degrading pectic enzymes. The observation that such a highly pectolytic organism also produces a harpin suggests the fundamental importance of harpins in the pathogenicity of gram-negative bacteria. The observation that an $hrpN_{Ech}$::Tn*5-gusA1* mutation reduced the ability of a fully pectolytic strain of *E. chrysanthemi* to initiate lesions in susceptible chicory leaves, but did not reduce the size of lesions that did develop, suggests that $HrpN_{Ech}$ contributes specifically to an early stage of pathogenesis. An attractive possibility is that $HrpN_{Ech}$ releases nutrients to the apoplast for bacterial nutrition before the *pel* genes are fully expressed (Collmer and Bauer 1994). Patterns of *pel* and $hrpN_{Ech}$ expression *in planta* will likely yield further clues to the role of the *E. chrysanthemi* harpin in soft-rot pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

Bacterial stains and plasmids are listed in Table 3. *E. chrysanthemi* was routinely grown in King's medium B (King *et al.* 1954) at 30° C, *E. coli* in LM medium (Hanahan 1983) at 37° C, and *E. amylovora* in Luria-Bertani medium at 28–30° C. The following antibiotics were used in selective media in the amounts indicated (in μg/ml), except where noted: ampicillin (100), kanamycin (50), spectinomycin (50), and streptomycin (25).

General DNA manipulations.

Plasmid DNA manipulations, colony blotting, and Southern blot analyses were performed by standard techniques (Sambrook *et al.* 1989). Deletions for sequencing were constructed with the Erase-a-Base kit (Promega, Madison, WI). Double-stranded DNA sequencing templates were prepared with Qiagen Plasmid Mini Kits (Chatsworth, CA). Sequencing was performed with the Sequenase Version 2 kit (U.S. Biochemical, Cleveland, OH). The Tn*5-gusA1* insertion points were determined on an Automated DNA Sequencer (model 373A, Applied Biosystems, Foster City, CA) by the Cornell Biotechnology Center. DNA sequences were analyzed with the Genetics Computer Group Sequence Analysis Software Package (Devereaux *et al.* 1984). Comparison of $HrpN_{Ech}$ and $HrpN_{Ea}$ by the Gap program was done with a gap weight of 5.0 and a gap length weight of 0.3. Marker exchange mutagenesis was performed as previously described (Bauer *et al.* 1994). The oligonucleotide used to determine the location of Tn*5-gusA1* insertions in $hrpN_{Ech}$ was TGACCTGCAGCCAAGCTTTCC. The oligonucleotide used as the first primer to amplify the $hrpN_{Ech}$ ORF and introduce an *Nco*I site at the 5′ end of the gene was AGTACCATGGTTATTACGATCAAAGCGCAC; the one used as the second primer to introduce an *Xho*I site at the 3′ end of the gene was AGATCTCGAGGGCGTTGGCCAGCTTACC. Primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Protein manipulations.

$HrpN_{Ech}$ was purified from *E. coli* DH5α(pCPP2172) cultures grown at 30° C to stationary phase in 50 ml of Terrific Broth (Sambrook *et al.* 1989) supplemented with ampicillin at a concentration of 200 μg/ml. Cells were lysed by lysozyme treatment and sonication as previously described (Sambrook *et al.* 1989). The lysate pellet was washed twice with 9 vol of lysis buffer containing 0.5% Triton X-100 and 10 mM EDTA, pH 8.0, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF); the lysate was reharvested each time by centrifugation at 12,000 × *g* for 15 min. The pellet was resuspended in 2.0 ml of lysis buffer containing 0.1 mM PMSF, dissolved by the addition of 2.5 ml of 8 M guanidine-HCl in lysis buffer, and then diluted with 5.0 ml of water. The protein solution was dialyzed in SpectraPor #1 dialysis tubing against 2.0 liters of 5 mM morpholinoethanesulfonic acid (MES), pH 6.5, containing 0.05 mM PMSF. The precipitate that formed dur-

ing dialysis and the solution were centrifuged for 15 min at 4,300 × g. The pellet was washed once with 10 ml of a solution containing 5 mM MES, pH 6.5, and 0.1 mM PMSF and then resuspended in 2.0 ml of the same buffer. Protein concentrations of homogeneous suspensions were determined following dissolution in the reagents of the dye-binding assay of Bradford (1976). Proteins in crude cell lysates or following purification were resolved by electrophoresis through an SDS 12% polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue R. The N-terminal sequence of purified $HrpN_{Ech}$ was determined at the Cornell University Biotechnology Program Protein Analysis Facility.

Plant assays.

For HR assays, tobacco (*Nicotiana tabacum* L. cv. Xanthi), tomato (*Lycopersicon esculentum* Mill. cv. Sweet 199), pepper (*Capsicum annuum* L. cv. Sweet Hungarian), African violet (*Saintpaulia ionantha* H. Wendl. cv. Paris), petunia (*Petunia grandiflora* Juss. cv. Blue Frost), pelargonium (*Pelargonium hortorum* Bailey), winter squash (*Cucurbita maxima* Duchesne), and zinnia (*Zinnia elegans* Jacq.) plants were grown under greenhouse conditions or purchased at a local garden shop and then maintained in the laboratory at room temperature, with incident daylight supplemented with a 500-W halogen lamp. Witloof chicory (*Cichorium intybus* L.) was purchased as "Belgian endive" heads from a local supermarket. Bacterial inoculum was prepared and delivered as previously described (Bauer *et al.* 1994). Briefly, to assay soft-rot pathogenesis, 5 µl of inoculum was applied to a small wound in detached chicory leaves; to assay for HR elicitation, inoculum was infiltrated with a needle-less plastic syringe into leaves on plants.

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The RsmA⁻ Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress $hrpN_{Ecc}$ and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves

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Erwinia carotovora subsp. *carotovora* wild-type strain Ecc71 does not elicit the hypersensitive reaction (HR) in tobacco leaves. By mini-Tn*5*-Km and chemical mutagenesis we have isolated RsmA⁻ mutants of Ecc71 that produce high basal levels of pectate lyases, polygalacturonase, cellulase, and protease; they also are hypervirulent. The RsmA⁻ mutants, but not their parent strains, elicit an HR-like response in tobacco leaves. This reaction is characterized by the rapid appearance of water soaking followed by tissue collapse and necrosis. The affected areas remain limited to the region infiltrated with bacterial cells, and the symptoms closely resemble a typical HR, e.g., the reactions caused by *Pseudomonas syringae* pv. *pisi*. Moreover, low concentrations of cells of the mini-Tn*5*-Km insertion RsmA⁻ mutant, AC5070, infiltrated into tobacco leaf tissue prevent elicitation of the rapid necrosis by AC5070 or by *P. syringae* pv. *pisi*. Elicitation of the HR-like response by the mutants is not affected by the deficiency of *N*-(3-oxohexanoyl)-L-homoserine lactone, the cell density (quorum) sensing signal. Cloning and sequence analysis have disclosed that *E. carotovora* subsp. *carotovora* strain Ecc71 possesses a homolog of *E. chrysanthemi hrpN* known to encode an elicitor of the HR; the corresponding Ecc71 gene is designated $hrpN_{Ecc}$. Northern (RNA) blot data show that the level of $hrpN_{Ecc}$ mRNA is considerably higher in the RsmA⁻ mutants than in the RsmA⁺ strains. Moreover, a low copy plasmid carrying the *rsmA*⁺ allele severely reduces the level of the $hrpN_{Ecc}$ transcripts in the RsmA⁻ mutants. These constructs, like the RsmA⁺ *E. carotovora* subsp. *carotovora* strains, do not elicit the HR-like response. These data taken along with the effects of *rsmA* on exoenzyme production and pathogenicity (A. Chatterjee et al., 1995, Appl. Environ. Microbiol. 61:1959-1967) demonstrate that this global regulator gene plays a critical role in plant interaction of *E. carotovora* subsp. *carotovora*.

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Nucleotide and/or amino acid sequence data is to be found at GenBank as accession number L78834.

Additional keywords: derepressed mutant, incompatible interactions, soft-rotting bacteria.

Many gram-negative phytopathogenic bacteria, when infiltrated into a nonhost plant such as tobacco, cause localized necrosis, generally known as the hypersensitive reaction (HR) (Goodman and Novacky 1994). A typical HR is characterized by the rapid collapse of the leaf tissue followed by necrosis of the collapsed area. *Erwinia carotovora* subsp. *carotovora* and many other soft-rotting bacteria are unusual in that they do not elicit a typical HR when infiltrated into tobacco leaves. The inability of these bacteria to elicit the HR has been attributed to the production of pectolytic enzymes that are presumed to suppress the HR. The recent finding of Collmer and his associates that a mutant strain of *E. chrysanthemi* deficient in the synthesis of the major pectate lyase (Pel) isozymes, but not the pectolytic parent, can elicit the HR (Bauer et al. 1994) is certainly consistent with this hypothesis. In fact, both genetic and biochemical data (Bauer et al. 1995) demonstrate that *E. chrysanthemi*, like many other gram negative bacteria, possesses *hrp* genes including *hrpN*, which encodes an elicitor of the HR. These data and the results of Southern blot hybridizations of Laby and Beer (1992) support the idea that soft-rotting *Erwinia* possess *hrp* genes, but a sustained expression of *hrp* genes of these *Erwinia* species in incompatible hosts may not occur at a level required for elicitation of the HR.

We have initiated studies to clarify the genetic regulation of the production of the HR and disease symptoms by *E. carotovora* subsp. *carotovora*. We previously reported that a mini-Tn*5*-Km insertion RsmA⁻ mutant of *E. carotovora* subsp. *carotovora* is derepressed in extracellular enzyme production and it is hypervirulent (Chatterjee et al. 1995; Cui et al. 1995). A mutant of similar phenotype was also generated by chemical mutagenesis. The data presented here show that these mutants elicit responses in tobacco leaves that are similar to those in a typical HR and that they do not require the cell density sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHL) to cause this reaction. Additionally, our findings disclose the presence of a homolog of the $hrpN_{Ecc}$ gene in *E. carotovora* subsp. *carotovora* strain Ecc71 and show that expression of this gene is negatively controlled by *rsmA*.

RESULTS

RsmA⁻ mutants of *E. carotovora* subsp. *carotovora* elicit responses in tobacco leaves that resemble the HR.

Previously (Chatterjee et al. 1995; Cui et al. 1995), we have described the isolation procedure as well as some of the characteristics of *E. carotovora* subsp. *carotovora* strain AC5070, the mini-Tn*5*-Km insertion RsmA⁻ mutant (*rsm* = *r*egulator of *s*econdary *m*etabolites). Since AC5070 overproduces pectate lyases, polygalacturonases, protease, and cellulase, and is hypervirulent, it was of interest to examine the responses it could elicit in tobacco leaves, wherein wild-type *E. carotovora* subsp. *carotovora* does not cause tissue necrosis in 24 to 48 hr. As shown in Figure 1, cells of AC5070 infiltrated into tobacco leaves produced symptoms similar to those caused by *P. syringae* pv. *pisi*, known to elicit the HR. The lowest concentration of AC5070 that elicited an HR-like response was approximately 2×10^8 cells/ml. The visible symptoms, i.e., water soaking followed by tissue collapse, appeared within 24 h after the infiltration. By 24 h the inoculation sites developed necrosis, culminating in tissue desiccation. These responses, as in the typical HR, invariably remained confined to the area infiltrated with bacterial cells. Infiltration with cells of RsmA⁺ *E. carotovora* subsp. *carotovora* grown in Luria-Bertani (LB) agar did not produce visible lesions; however, after 5 to 6 days the infiltrated sites became chlorotic.

By ethyl methane sulfonate (EMS) mutagenesis of *E. carotovora* subsp. *carotovora* strain AC5006, we isolated a mutant, AC5041, that, like AC5070, overproduces pectate lyases, polygalacturonases, protease, and cellulase (Fig. 2). In addition, the mutant is hypervirulent in that it caused more severe maceration in celery petioles than the parent RsmA⁺ strain (Fig. 3). The derepressed mutant, AC5041, but not its parent strain, induced the HR-like response in tobacco leaves (data not shown).

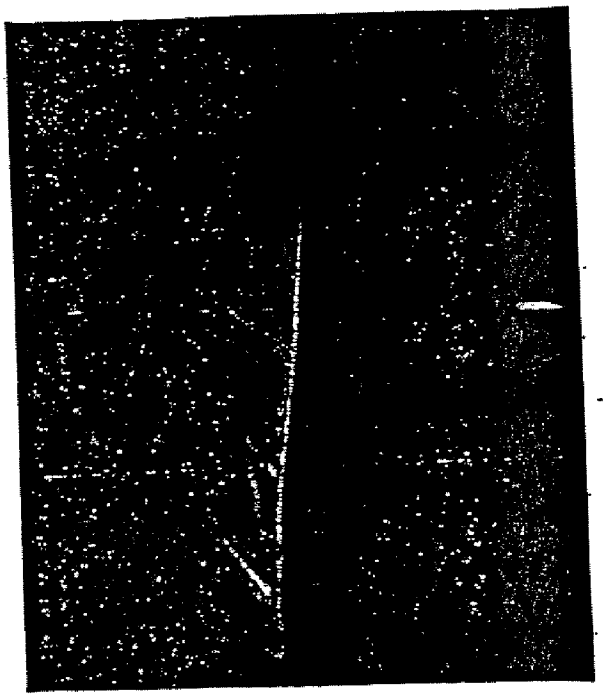

Fig. 1. Symptoms produced in tobacco leaves by *Erwinia carotovora* subsp. *carotovora* AC5047 and its RsmA⁻ mutant, AC5070. Cell suspensions containing about 2×10^8 CFU/ml were infiltrated into each leaf segment. A, AC5047; B, AC5070; C, *Pseudomonas syringae* pv. *pisi* Psp1; and D, water. Picture was taken 24 h after infiltration.

Prevention of the HR-like response.

It has been reported that *P. syringae* pv. *pisi* prevents the HR when it is preinoculated in tobacco leaves at a lower concentration (5×10^5) and later challenged with an HR-inducing concentration (5×10^6) at the same site (Novacky et al. 1973). Similarly, we have noticed that preinfiltration of tobacco leaves with AC5070 (10^5 CFU/ml) prevented the appearance of water soaking and necrosis upon reinoculation at the same

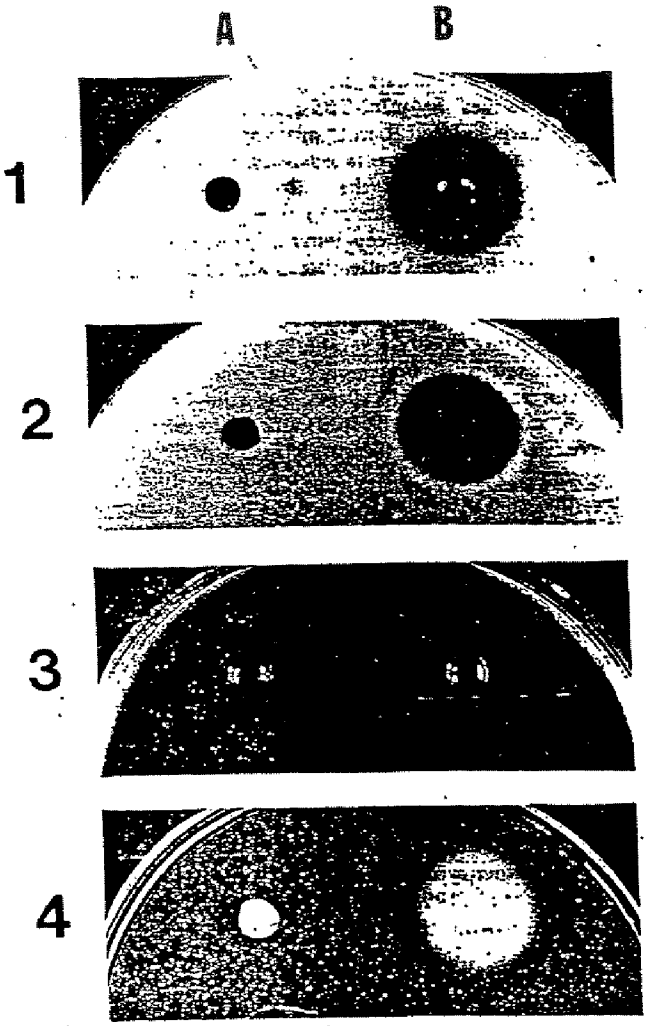

Fig. 2. Agarose plate assays for 1, pectate lyase (Pel); 2, polygalacturonase (Peh); 3, protease (Prt); and 4, cellulase (Cel) activities of *Erwinia carotovora* subsp. *carotovora* AC5006 (A) and its RsmA⁻ mutant AC5041 (B). Bacteria were grown in salts–yeast extract–glycerol medium to saturation. Culture supernatants were diluted twofold in 10 mM Tris-HCl (pH 7.0) buffer and 5 µl of the diluted samples were used for the Pel, Peh, and Cel assays. Thirty microliters of undiluted samples were used for the Prt assay.

site with AC5070 or *P. syringae* pv. *pisi* (Fig. 4). After the preinoculation, about 2×10^8 cells of AC5070 were introduced at different intervals. The ability of preinoculated cells to inhibit the HR-like response was apparent by 12 h after inoculation (data not shown), and by 24 h production of the response was completely suppressed.

Fig. 3. Maceration of celery petioles induced by *Erwinia carotovora* subsp. *carotovora* AC5006 (A) and its RsmA⁻ mutant AC5041 (B). About 2×10^8 bacterial cells suspended in water were injected into each inoculation site. Inoculated petioles were covered with petroleum jelly and incubated in a moist chamber at 25°C for 24 h.

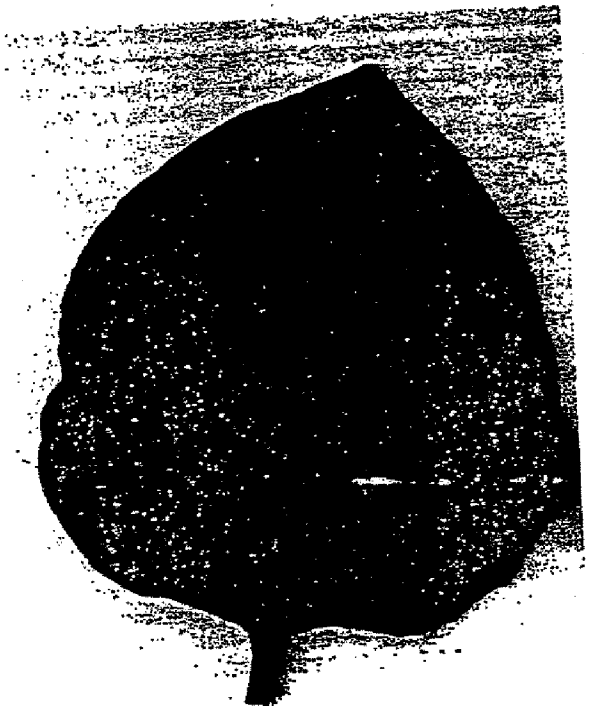

Fig. 4. Prevention of the hypersensitive response symptoms in tobacco leaf by the RsmA⁻ mutant of *Erwinia carotovora* subsp. *carotovora*, AC5070. Leaf segments were infiltrated with A, water at 0 h; B, *Pseudomonas syringae* pv. *pisi* Psp1 (5×10^8 CFU/ml) at 24 h; C, AC5070 (2×10^8 CFU/ml) at 24 h; D, AC5070 (2×10^8 CFU/ml) at 0 h; E, AC5070 (10^5 CFU/ml) at 0 h; F, AC5070 (10^5 CFU/ml) at 0 h and challenged with Psp1 (5×10^8 CFU/ml) after 24 h; G, AC5070 (10^5 CFU/ml) at 0 h and challenged with AC5070 (2×10^8 CFU/ml) after 24 h; and H, Psp1 (5×10^8 CFU/ml) at 0 hour. Leaf was photographed 48 h after infiltration.

RsmA⁻ mutants of *E. carotovora* subsp. *carotovora* elicit the HR-like response in the absence of the cell density sensing signal, OHL.

OHL and its structural analogs are required for the expression of many phenotypes in different bacteria (Fuqua et al. 1994; Salmond et al. 1995; Swift et al. 1994). In *E. carotovora* subsp. *carotovora*, OHL controls extracellular enzyme production, pathogenicity, and production of the antibacterial antibiotic, carbapenem (Bainton et al., 1992; Chatterjee et al. 1995; Jones et al. 1993; Pirhonen et al. 1993). We had previously demonstrated that exoenzyme overproduction and pathogenicity occurred in the absence of OHL in the RsmA⁻ mutant, AC5070 (Chatterjee et al. 1995). To find out if the mutants could elicit the HR-like response in the absence of this cell density sensing signal, we examined the responses induced by OHL-deficient derivatives of the RsmA⁻ strains. We made the EMS-induced RsmA⁻ mutant OHL deficient by replacing *ohlI*⁺ (previously designated as *hslI*⁺) allele required for OHL biosynthesis, with *ohlI*-MudI by marker exchange, as we had done with AC5070 (Chatterjee et al. 1995). AC5090 and AC5093, the derivatives of AC5070 and AC5041, respectively, do not produce OHL, as indicated by the Lux bioassay (Chatterjee et al. 1995; data not shown). Figure 5 shows that AC5090 and AC5093 elicited reactions in tobacco leaves that were very similar to those produced by the parent strains as well as by *P. syringae* pv. *pisi*.

The RsmA⁻ mutants overexpress $hrpN_{Ecc}$, a locus presumed to specify an elicitor of the HR.

Recent studies by S. V. Beer, A. Collmer, and their associates demonstrated that *hrpN* genes of *E. amylovora* and *E. chrysanthemi* encode elicitors of the HR and raised the possi-

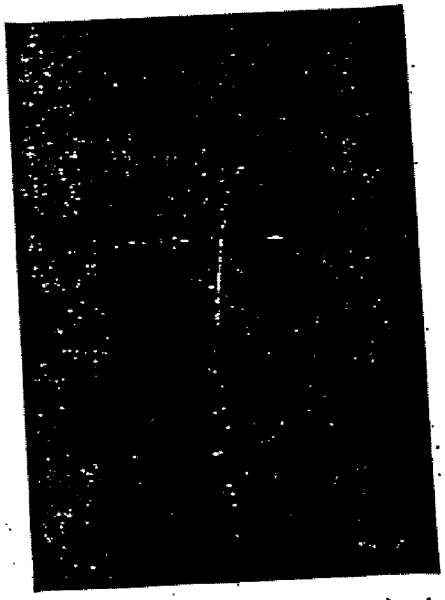

Fig. 5. Elicitation of the hypersensitive-like response in tobacco leaves by RsmA⁻ mutants of *Erwinia carotovora* subsp. *carotovora* and their OhlI⁻ derivatives. Leaf segments were infiltrated with 2×10^8 CFU/ml of bacterial cells. A, water; B, AC5093 (RsmA⁻, OhlI⁻); C, AC5090 (RsmA⁻, OhlI⁻); D, *Pseudomonas syringae* pv. *pisi* Psp1; E, AC5041 (RsmA⁻, OhlI⁺); and F, AC5070 (RsmA⁻, OhlI⁺). Picture was taken 24 h after infiltration.

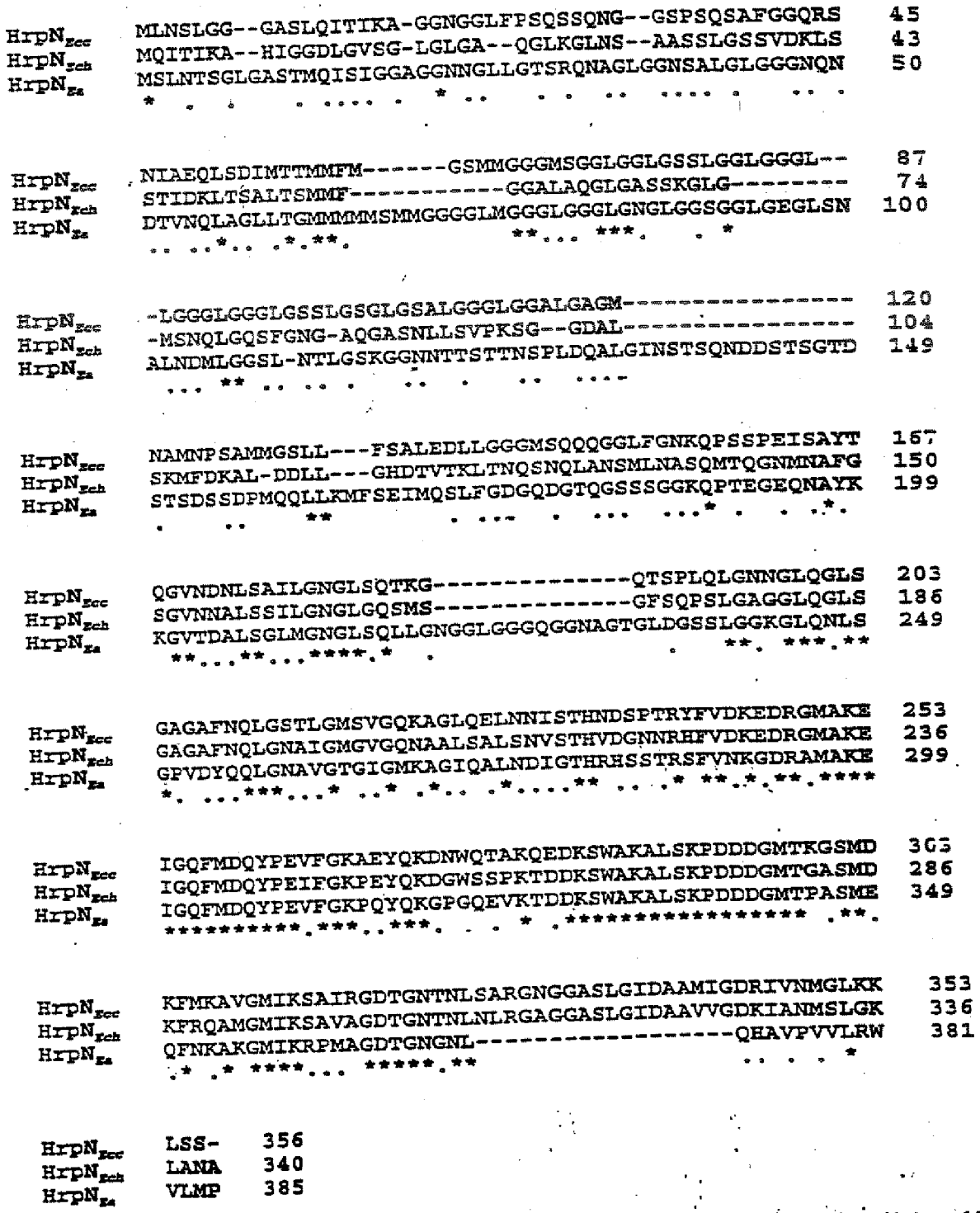

Fig. 6. Alignment of deduced amino acid sequence of *hrpN*$_{Ecc}$ of *Erwinia carotovora* subsp. *carotovora* strain Ecc71 (HrpN$_{Ecc}$) with those of *E. chrysanthemi* EC16 (HrpN$_{Ech}$) and *E. amylovora* Ea321 (HrpN$_{Ea}$). Asterisks indicate identical amino acids; single dots indicate conservative substitutions. Numbers at R right indicate amino acid positions in each protein.

bility that *hrp* genes including *hrpN* may also occur in other *Erwinia* species (Bauer et al. 1994; Bauer et al. 1995; Laby and Beer 1992; Wei et al. 1992). Indeed, Southern blot hybridization under moderate stringency conditions with *hrpN* DNA of *E. chrysanthemi* (EC16) (Bauer et al. 1995) as the probe disclosed the presence of *hrpN* sequences in *E. carotovora* subsp. *carotovora* strain Ecc71 (data not shown). Subsequently, by screening a library of Ecc71 with the *hrpN* DNA of *E. chrysanthemi*, several clones possessing homologous DNA were identified; the corresponding Ecc71 sequences are tentatively designated as $hrpN_{Ecc}$. Sequence analysis of the DNA segment that specifically hybridized with the *hrpN* DNA of *E. chrysanthemi* revealed an 1,068-bp open reading frame whose predicted product has 72.1% similarity and 53.4% identity with the deduced product of *hrpN* of *E. chrysanthemi*, and 66.6% similarity and 50.8% identity with the predicted product of *hrpN* of *E. amylovora* (Fig. 6).

Northern (RNA) blot analysis was performed with total RNA preparations from the wild-type strain Ecc71, the $RsmA^-$ mutants, AC5041 and AC5070, and their $RsmA^+$ parents to ascertain if $hrpN_{Ecc}$ expression is derepressed in the $RsmA^-$ strains. Bacteria were grown in SYG medium at 28°C to a Klett value of approximately 200 and used for total RNA isolation. A 700-bp *Acc*I-*Sma*I internal fragment of the $hrpN_{Ecc}$ was used as the probe. The data (Fig. 7) revealed the presence of 1100-base transcripts in AC5070 and AC5041. By contrast, these transcripts were not detected with $RsmA^+$ strains 71, AC5006 and AC5047. We should note that somewhat higher levels of $hrpN_{Ecc}$ transcripts were present in the mini-Tn*5*-Km insertion mutant (AC5070) than in the EMS-induced mutant (AC5041). We do not yet know the reason for this difference. It is possible that AC5041 produces a defective RsmA with a leaky activity, whereas the mini-Tn*5*-Km insertion mutant does not produce a functional RsmA. It is, however, clear that $hrpN_{Ecc}$ transcripts are substantially higher in AC5041 than in its $RsmA^+$ parent, AC5006.

The $rsmA^+$ allele suppresses elicitation of the HR-like response and expression of $hrpN_{Ecc}$

We have previously described the cloning and characterization of the *rsmA* gene of *E. carotovora* subsp. *carotovora* strain Ecc71 (Chatterjee et al. 1995; Cui et al. 1995). A low-copy plasmid carrying this gene causes a severe attenuation of pathogenicity and suppresses extracellular enzyme production in *E. carotovora* subsp. *carotovora* and *E. c.* subsp. *atroseptica*; represses pathogenicity, exopolysaccharide production, flagellum production and motility, protease production, and elicitation of the HR by *E. amylovora*; and suppresses extracellular enzyme and antibiotic production by *E. carotovora* subsp. *betavasculorum* (Chatterjee et al. 1995; Cui et al. 1995; Mukherjee et al. 1996a, 1996b). In light of the large array of effects on phenotypes by *rsmA*, including induction of the HR by *E. amylovora*, it was deemed worthwhile to examine the effects of the $rsmA^+$ DNA on elicitation of the HR-like response by the mutants. The plasmids pCL1920 and pAKC880 were transformed into AC5041 and AC5070 and the constructs were tested for induction of the HR-like response. Figure 8 shows that AC5041 and AC5070 carrying the cloning vector, pCL1920, elicited reactions in tobacco leaves similar to those caused by *P. syringae* pv. *pisi*. By contrast, there was no visible reaction in the leaf segment infiltrated with AC5041 or AC5070 carrying the $RsmA^+$ plasmid, pAKC880. These results indicate that multiple copies of *rsmA* suppress elicitation of the HR-like response in tobacco leaves by AC5041 and AC5070.

Northern analysis was conducted to determine the effect of RsmA plasmid on $hrpN_{Ecc}$ transcription. The data (Fig. 9) show that high levels of $hrpN_{Ecc}$ transcripts were present in cells of AC5041 and AC5070 containing the cloning vector, pCL1920, but the transcripts were not detected in cells carrying the *rsmA* plasmid, pAKC880.

DISCUSSION

We previously reported that extracellular enzyme production as well as virulence are negatively regulated by *rsmA* in *E. carotovora* subsp. *carotovora* (Chatterjee et al. 1995; Cui et al. 1995; Mukherjee et al. 1996a, 1996b). For example, the inactivation of *rsmA* by a transposon resulted in overproduction of extracellular enzymes and hypervirulence. Moreover, unlike its parent, the $RsmA^-$ mutant did not require the cell density sensing signal, OHL, for pathogenesis or extracellular enzyme production. In this report, we have shown that this $RsmA^-$ mutant and an EMS-induced mutant of a similar phenotype elicited the HR-like response in tobacco leaves, and that the elicitation of this reaction was also not dependent upon OHL. Although we do not yet have direct evidence that the mutations in AC5041 and AC5070 are in the same gene, these strains possess similar phenotypes; e.g., they overproduce extracellular enzymes, they are hypervirulent, and OHL deficiency does not affect the expression of these traits. Moreover, the plasmid carrying $rsmA^+$ DNA suppresses extracellular enzyme production, pathogenicity, and the elicitation

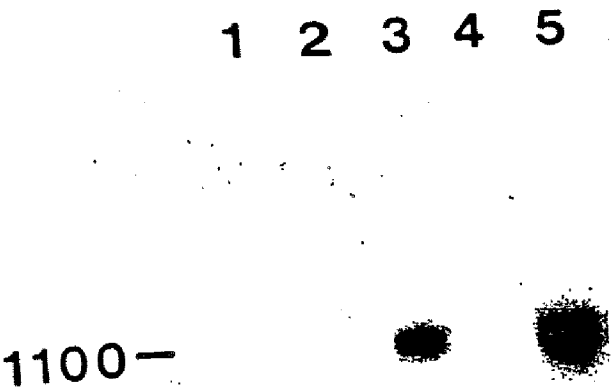

Fig. 7. Northern (RNA) blot analysis of $hrpN_{Ecc}$ mRNA of *Erwinia carotovora* subsp. *carotovora* strains. Each lane contained 20 µg of total RNA. Position of 1100-base transcript is indicated. Lane 1, Ecc71 (wild-type parent, $RsmA^+$); lane 2, AC5006 ($RsmA^+$); lane 3, AC5041 ($RsmA^-$); lane 4, AC5047 ($RsmA^+$); lane 5. AC5070 ($RsmA^-$).

of the HR-like response by the mutants. Also, both the mutants express $hrpN_{Ecc}$ constitutively, although the transcript level is somewhat higher in AC5070 than in AC5041. As these mutants have similar phenotypes, we tentatively classified them as RsmA$^-$.

The following lines of evidence strongly suggest that the mutants elicited a typical HR (Goodman and Novacky 1994): (i) the reaction was characterized by a rapid physiological activity (i.e., water movement or water soaking), tissue collapse followed by cell death (necrosis); (ii) the affected areas were limited to the region infiltrated with bacterial cells; (iii) these symptoms were indistinguishable from the symptoms developed by *P. syringae* pv. *pisi*, a bacterium known to elicit the typical HR in tobacco leaves; (iv) the response elicited by AC5070 was preventable upon previous infiltration of a low concentration of AC5070 cells and, similarly, prior inoculations with AC5070 cells prevented elicitation of the HR by *P. syringae* pv. *pisi*; and (v) while AC5070 and AC5041, their parent strains, and the wild-type strain possess $hrpN_{Ecc}$ sequences (data not shown), the expression of $hrpN_{Ecc}$ is derepressed only in the mutants, presumably leading to the production of high levels of a putative elicitor of the HR (see below).

Our observations support the idea that AC5070 and AC5041 produce an elicitor that triggers the HR-like response in tobacco leaves. We attribute the manifestation of this response with the mutants, but not with the parents, to the ability of the former to produce high constitutive levels of $HrpN_{Ecc}$, an exoenzyme, or both. With regard to the possible role of exoenzymes, it is perhaps significant that pectinases are known to generate elicitors of plant defense responses (Davis et al. 1984; Davis and Ausubel 1989; Keen 1992). Furthermore, Palva et al. (1993) have documented the activation of chitinases and glucanases in tobacco by exoenzyme-producing strains of *E. carotovora* subsp. *carotovora* but not by mutants deficient in exoenzyme production. Therefore, one could argue that pectinase overproduction by the RsmA$^-$ mutants may induce defense reactions that could culminate in an HR-like response. The inability of the wild-type RsmA$^+$ *E. carotovora* subsp. *carotovora* strain Ecc71 to elicit this response could be attributed to the lack of extracellular enzyme production in a nonhost tissue, i.e., in a tobacco leaf. However, the hypothesis implicating pectolytic enzymes as elicitors of the HR is difficult to reconcile with the finding of Bauer et al. (1994) that only those mutants of *E. chrysanthemi* that are deficient in major pectate lyases can elicit the HR.

In light of that finding and for the following reasons, we favor the hypothesis that induction of the HR-like response by the mutants may be due to the derepression of a gene encoding an elicitor, such as $HrpN_{Ech}$ or $HrpN_{Es}$. Collmer and asso-

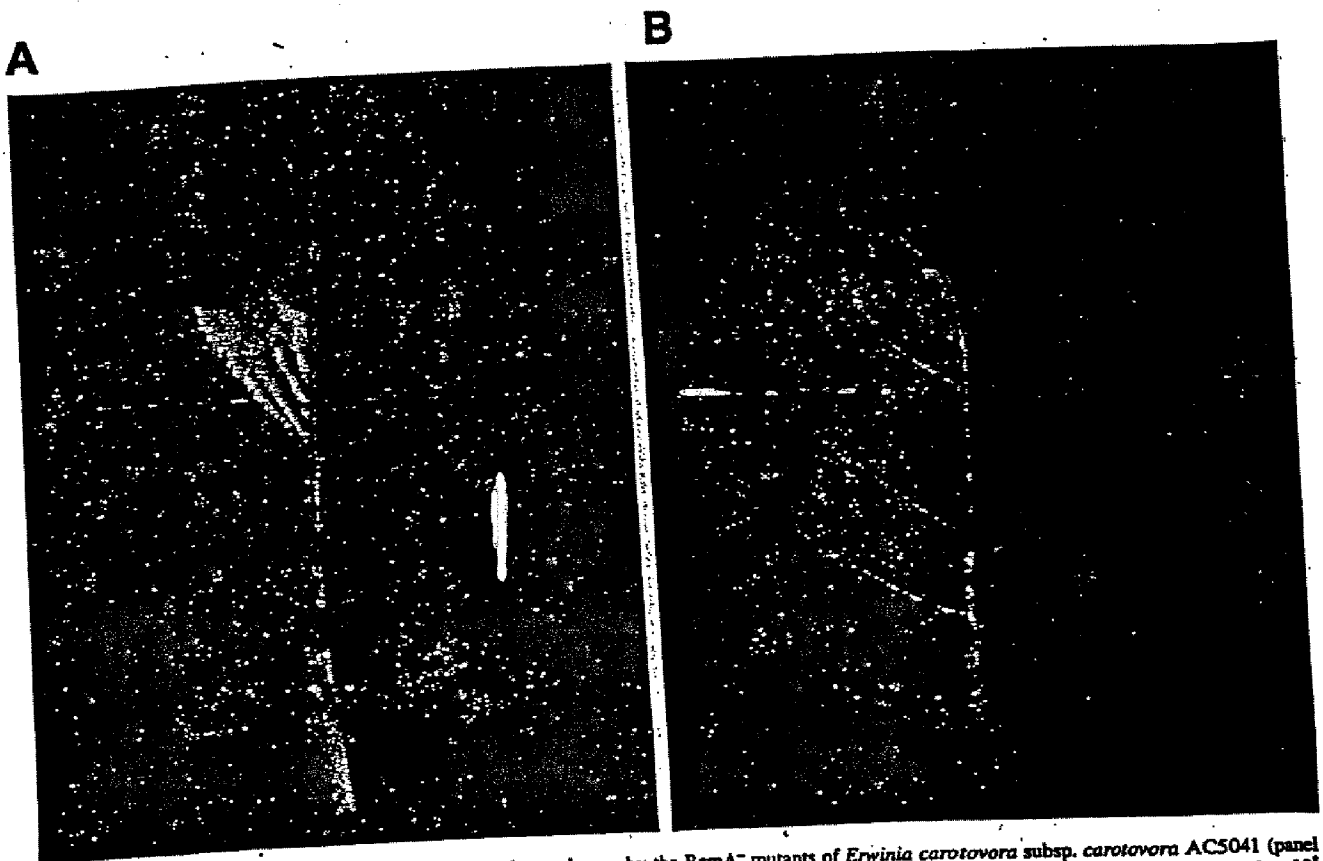

Fig. 8. Elicitation of an hypersensitive-like response in tobacco leaves by the RsmA$^-$ mutants of *Erwinia carotovora* subsp. *carotovora* AC5041 (panel A) and AC5070 (panel B) carrying the *rsmA*$^+$ plasmid, pAKC880, or the cloning vector, pCL1920. Bacterial suspensions containing about 2×10^8 CFU/ml were infiltrated into each leaf segment. Panel A: A, *Pseudomonas syringae* pv. *pisi* Psp1; B, AC5041 carrying pAKC880; C, water; D, AC5041 carrying pCL1920. panel B: A, Psp1; B, AC5070 carrying pAKC880; C, water; D, AC5070 carrying pCL1920. Picture was taken 24 h after infiltration.

ciates (Bauer et al. 1994; Bauer et al. 1995) have discovered a gene specifying an elicitor of the HR in the soft-rotting bacterium *E. chrysanthemi*. The deduced sequence of $HrpN_{Ecc}$ presented here document the occurrence of a homolog of *E. chrysanthemi hrpN* in *E. carotovora* subsp. *carotovora* strain Ecc71. We have found that the mini-Tn*5*-Km induced $RsmA^-$ mutant as well as the EMS-induced derepressed mutant possess a substantial level of an approximately 1100-base transcript that specifically hybridizes with the $hrpN_{Ecc}$ DNA. By contrast, this transcript is barely detectable in the $RsmA^+$ strains. Moreover, the introduction of the $rsmA^+$ allele into the mutants severely reduces the levels of this transcript and concomitantly abolishes the ability to elicit the HR-like response. These observations indicate that transcription of $hrpN_{Ecc}$ is derepressed in the mutants, and that this derepression is due to the inactivation of *rsmA*. At the moment, since the genes for pectolytic enzymes and $hrpN_{Ecc}$ are both derepressed in the $RsmA^-$ mutants, we have to entertain the possibility that the pectolytic enzymes could also contribute to the hypersensitive necrosis of tobacco leaf tissue. Genetic and biochemical studies have been initiated to determine if $hrpN_{Ecc}$ and its putative product are solely responsible for the elicitation of the HR and to clarify the ramifications of $hrpN_{Ecc}$ regulation in compatible and incompatible interactions of *E. carotovora* subsp. *carotovora*.

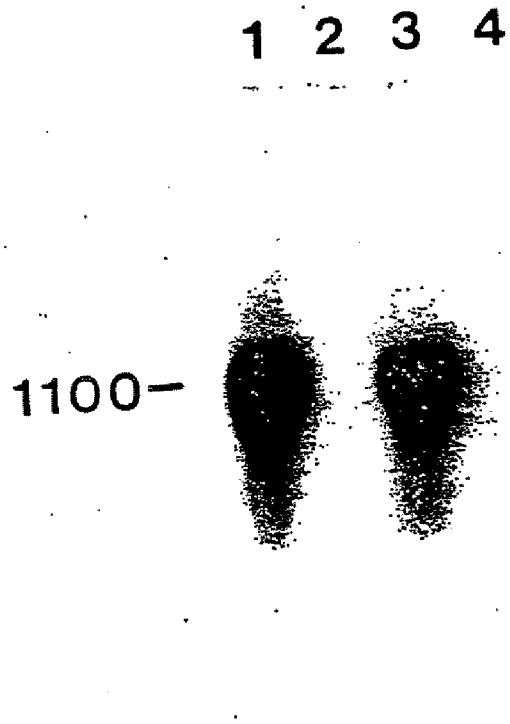

Fig. 9. Northern (RNA) blot analysis of $hrpN_{Ecc}$ mRNA of *Erwinia carotovora* subsp. *carotovora* $RsmA^-$ mutants AC5041 and AC5070 carrying the $rsmA^+$ plasmid, pAKC880, or the cloning vector, pCL1920. Each lane contained 20 µg of total RNA. The position of 1100-base transcript is indicated. Lane 1, AC5070 carrying pCL1920; lane 2, AC5070 carrying pAKC880; lane 3, AC5041 carrying pCL1920; lane 4, AC5041 carrying pAKC880.

MATERIALS AND METHODS

Bacterial strains and media.

Bacterial strains and plasmids are described in Table 1. *E. carotovora* subsp. *carotovora* strains were routinely grown in LB and *P. syringae* pv. *pisi* on King's B (King et al. 1954) agar media at 28°C. Minimal salts plus sucrose (0.2%) agar, nutrient gelatin (NG) agar, polygalacturonate-yeast extract agar (PYA) and salts-yeast extract-glycerol (SYG) media have been described previously (Barras et al. 1987; Chatterjee 1980; Murata et al. 1991). When required, antibiotics were added at the indicated concentrations in micrograms per milliliter: spectinomycin (Spc), 50; tetracycline (Tc), 10; Ampicillin (Ap), 50 and Kanamycin (Km), 50. The composition of agarose media for semiquantitative assays of enzymatic activities has been described in Chatterjee et al. (1995).

Enzyme assays.

The preparation of enzyme samples for assays as well as the assay procedures were described previously (Murata et al. 1991; Chatterjee et al. 1995). The volumes of enzyme samples used in the assays are indicated in the figure legends.

Bioluminescence assay for OHL.

The procedure described by Chatterjee et al. (1995) was followed.

Recombinant DNA techniques.

Standard procedures were followed in DNA isolation, transformation and electroporation of bacteria, restriction digests, gel electrophoresis, DNA ligation, and Southern blot analysis (Sambrook et al. 1989). Restriction and modifying enzymes were obtained from Promega Biotech (Madison, WI).

Isolation of $RsmA^-$ mutants.

The procedure used for the isolation of AC5070 by mini-Tn*5*-Km has been described (Chatterjee et al. 1995). AC5041 was isolated by EMS mutagenesis of AC5006. Mutagenesis was carried out according to the protocol of Miller (1972). The bacterial cells were incubated with EMS for a period that yielded less than 5% survival. The putative $RsmA^-$ mutants were identified by their ability to overproduce protease, cellulase, and pectolytic enzymes in agar plate assays (Chatterjee et al. 1995).

Inactivation of the *ohl* locus by MudI mutagenesis.

The plasmid, pAKC852, carrying the 9.7-kb ohl^+ DNA of *E. carotovora* subsp. *carotovora* strain Ecc71 was mutagenized with MudI1734 following the procedure of Castilho et al. (1984). Briefly, pAKC852 was transformed into the lysogenic *Escherichia coli* strain POI1734. The strain carrying the Ohl^+ plasmid was heat-induced to lyse. The lysate was used to transduce *E. coli* M8820, and the Tc^rKm^r transductants were screened for OHL production by means of the plate assay procedure described in Chatterjee et al. (1995). Plasmids were isolated from M8820 colonies that could no longer activate the *lux* operons in pHV200L.

Construction of bacterial strains by marker exchange.

The construction of AC5090, the Ohl^- derivative of AC5070, has been described (Chatterjee et al. 1995). To isolate AC5093, the Ohl^- mutant of AC5041, the plasmid (pAKC863) carrying inactivated *ohlI*-MudI was transferred into AC5041 by means of the helper plasmid, pRK2013.

Transconjugants were selected on minimal salts plus sucrose agar supplemented with Km. Colonies that were Km^rTc^s were tested for the Ohl phenotype. AC5093 was selected for further studies.

Plant tissue maceration.

The celery petiole assay was previously described (Murata et al. 1991). The extent of tissue maceration was estimated visually.

Infiltration of tobacco leaves.

Erwinia species were grown on LB agar and *P. syringae* pv. *pisi* was grown on King's B agar overnight at 28°C and cells were resuspended in water. Strains carrying plasmids were grown on LB agar containing spectinomycin and cells suspended in a 50 µg/ml spectinomycin solution in water. Young, fully expanded third and fourth leaves of about 8-week-old *Nicotiana tabacum* L. cv. Samsun were infiltrated with bacterial suspensions. Inoculated plants were incubated in a growth chamber at 27°C with a 14/10 h daylight regime and visually monitored for reactions. For testing the prevention of the HR-like response, cells of AC5070 (10^5 CFU/ml) were infiltrated into tobacco leaves. The preinoculated areas were reinoculated with 2×10^8 CFU of AC5070 per ml or 5×10^6 CFU of *P. syringae* pv. *pisi* Psp1 per ml at desired intervals.

Cloning of $hrpN_{Ecc}$ DNA and nucleotide sequence analysis.

The genomic library of *E. carotovora* subsp. *carotovora* strain Ecc71 in pLARF5 was screened by in situ colony hybridization with a 0.75-kb internal *ClaI* fragment of *hrpN* of *E. chrysanthemi* (Bauer et al. 1995). Two cosmids, pAKC921 and pAKC922, that hybridized with the probe were isolated. The subclones (pAKC923 and pAKC924, Table 1) carrying *hrpN* DNA were used for sequence analysis.

Unidirectional 5' to 3' deletions of pAKC924 were made and the overlapping deletions differing in size by approximately 200 bp were used for sequence analysis with the Sequenase System II (U.S. Biochemicals, Cleveland, OH). In addition, we used oligonucleotide primers to verify and complete the sequence of $hrpN_{Ecc}$ with pAKC923 and pAKC924 DNAs as templates. Alignment of protein sequences was performed using the Genetics Computer Group, Inc. (Madison, WI) software program at the DNA Core facility on the University of Missouri-Columbia campus and the PC/GENE program (IntelliGenetics, Inc., Mountain View, CA). The sequence of $hrpN_{Ecc}$ has been deposited at GenBank and has been assigned accession number L78834.

Northern blot analysis.

Bacterial cultures were grown to a value of approximately 200 Klett units at 28°C in SYG medium with or without

Table 1. Bacterial strains and plasmids

Bacteria	Relevant characteristics[a]	Reference or source
Erwinia carotovora subsp. *carotovora*		
71	Wild type	Zink et al. 1984
AC5006	Lac^- mutant of 71	Murata et al. 1991
AC5041	$RsmA^-$, EMS mutant of AC5006	This study
AC5047	Nal^r derivative of AC5006	Chatterjee et al. 1995
AC5070	$RsmA^-$, mini-Tn*5*-Km mutant of AC5047, Km^r, Nal^r	Chatterjee et al. 1995
AC5090	Ohl^- derivative of AC5070, $RsmA^-$, Km^r, Spc^r	Chatterjee et al. 1995
AC5093	Ohl^- derivative of AC5041, $RsmA^-$, Km^r	This study
Pseudomonas syringae pv. *pisi*		
Psp1	Wild type	A. J. Novacky
Escherichia coli		
DH5α	*ϕ80lacZ ΔM15, Δ(lacZYA-argF), U169 hsdR17 recA1 endA1 thi-1*	BRL, Frederick, MD
HB101	*proA1 lacY hsdS20(rB⁻ mB⁻), recA56 rpsL20*	Zink et al. 1984
M8820	*Δ(proAB-argF-lacPOZYA)recA⁺*	Castilho et al. 1984
POI1734	MudI1734::ara(Mu cts), *Δ(proAB-argF-lacIPOZYA)*	Castilho et al. 1984
VIS533	*ara.Δ(lac-proAB) rpsL ϕ80lacZ. ΔM15 recA56*	Gray and Greenberg 1992
Plasmids		
pAKC852	$OhlI^+$, Tc^r	Chatterjee et al. 1995
pAKC863	Derived from pAKC852, *ohlI*::MudI, Km^r, Tc^r	This study
pAKC880	$RsmA^+$, Spc^r	Cui et al. 1995
pAKC921	pLARF5 containing $hrpN_{Ecc}$ from genomic library of Ecc71, Tc^r	This study
pAKC922	pLARF5 containing $hrpN_{Ecc}$ from genomic library of Ecc71, Tc^r	This study
pAKC923	4.0-kb *Eco*RI fragment of pAKC921 containing $hrpN_{Ecc}$ cloned into pSK^+, Ap^r	This study
pAKC924	1.4-kb *Eco*RI fragment of pAKC922 containing $hrpN_{Ecc}$ cloned into pSK^+, Ap^r	This study
pCL1920	Spc^r	Lerner and Inouye 1990
pCPP2172	$hrpN_{Ecc}$, Ap^r	Bauer et al. 1995
pLARF5	Tc^r	Keen et al. 1988
pRK415	Tc^r	Keen et al. 1988
pRK2013	Mob^+, Tra^+, Km^r	Figurski and Helinski 1979
pBluescript SK+	Ap^r	Stramgene, La Jolla, CA
pHV200	8.8-kb *lux* DNA in pBR322, Ap^r	Gray and Greenberg 1992
pHV200I	Frameshift mutation of luxI in pHV200, Ap^r	Pearson et al. 1994

[a] Uncommon abbreviations: EMS = ethyl methane sulfonate; Ohl = *N*-(3-oxohexanoyl)-L-homoserine lactone, designated as Hsl in our previous publications; *rsmA* = regulator of secondary metabolites; $hrpN_{Ecc}$ = *E. carotovora* subsp. *carotovora* DNA fragment carrying a $hrpN_{Ech}$ homolog (Bauer et al. 1995).

spectinomycin. The procedures for RNA isolation and Northern blot analysis described in Chatterjee et al. (1991) and Liu et al. (1993) were followed. A 0.7-kb *Acc*I-*Sma*I internal fragment of $hrpN_{Ecc}$ was used as the probe.

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HARPIN IS NOT NECESSARY FOR THE PATHOGENICITY OF *ERWINIA STEWARTII* ON MAIZE.

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Erwinia stewartii elicits a hypersensitive response (HR) in tobacco if expression of the *hrp*-like *wts* regulon is enhanced. A clone containing *E. amylovora* $hrpN_{Ea}$ was used as a hybridization probe to locate a gene for harpin production, $hrpN_{Es}$, within the *wts* gene cluster. Transposon mutagenesis and complementation analysis revealed that $hrpN_{Es}$ is a monocistronic operon. Sequence analysis indicated that it encodes a 382-amino acid, glycine-rich polypeptide, which lacks cysteine and an N-terminal signal peptide. $Harpin_{Es}$ is 58% identical and 78% homologous to $harpin_{Ea}$, and 41% identical and 66% homologous to $harpin_{Ech}$ from *E. chrysanthemi*. Purified $harpin_{Es}$ was protease sensitive and heat-stable, and it elicited a typical HR in tobacco leaves. Antibodies to $harpin_{Es}$ cross-reacted with $harpin_{Ea}$, and conversely. $Harpin_{Es}$ was found in cytoplasmic, membrane, and extracellular fractions. Chromosomal mutations in $hrpN_{Es}$ were constructed by Tn*5* mutagenesis and marker-exchange. The mutants were HR- and did not produce detectable harpin in Western blots. However, they remained fully pathogenic on maize seedlings with respect to symptom severity, ED_{50} and response time, and they grew as well as the wild-type strain *in planta*. Likewise, loss of harpin did not affect the ability of a $hrpN_{Es}$ mutant to grow endophytically in several grasses. *wtsB*, *wtsD*, and *wtsF* mutants accumulated $Harpin_{Es}$ intracellularly, indicating that these DNA regions are necessary for harpin secretion.

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Molecular differentiation of *Erwinia amylovora* strains from North America and of two Asian pear pathogens by analyses of PFGE patterns and *hrpN* genes

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Summary

In order to determine a possible genomic divergence of *Erwinia amylovora* 'fruit tree' and raspberry strains from North America, several isolates were differentiated by pulsed-field gel electrophoresis (PFGE) analysis, the size of short DNA sequence repeats (SSRs) and the nucleotide and deduced amino acid sequences of their *hrpN* genes. By PFGE analysis European strains are highly related, whereas strains from North America were diverse and were further distinguished by the SSR numbers from plasmid pEA29. The *E. amylovora* strains from Europe showed identical HrpN sequences in contrast to the American isolates from fruit trees and raspberry. Those were related to each other, but distinguishable by their HrpN patterns. The Asian pear pathogens differed in HrpN among each other and from *E. amylovora*. *Erwinia pyrifoliae* isolates and the *Erwinia* strains from Japan were ordered via their HrpN sequences in agreement with the PFGE patterns. For all three pathogens, dendrograms from PFGE and sequence data indicate an evolutionary diversity within the species in spite of a genetic conservation for parts of the *hrpN* genes suggesting a long persistence of the Asian pear pathogens in Korea and Japan as well as of fire blight in North America. Some of the divergent American *E. amylovora* isolates share PFGE patterns with the relatively uniform European strains.

Introduction

Fire blight of apple and pear fruit trees and raspberry as well as of other rosaceaous plants is assumed to have originated in the Eastern part of North America, from where the disease might have been endemic for a long time, and was then distributed in the last century to many countries of the Northern hemisphere and to New Zealand (Bonn and van der Zwet, 2000). In Korea, a bacterial disease of pears and its causative agent *Erwinia pyrifoliae* has been described (Rhim *et al.*, 1999), which was distinguished from *Erwinia amylovora* by molecular and microbiological tools (Kim *et al.*, 1999) and additional DNA sequences (McGhee *et al.*, 2002). Another disease, bacterial shoot blight of pear, was noticed on the island of Hokkaido in Japan (Beer *et al.*, 1996) and the pathogen has been shown to be more related to *E. pyrifoliae* than to *E. amylovora* (Kim *et al.*, 2001a).

Erwinia amylovora has been extensively investigated for many physiological, biochemical and molecular features (reviewed in Vanneste, 2000). Two main factors are a strict requirement for pathogenicity: the ability to produce the acidic exopolysaccharide (EPS) amylovoran, encoded in the 17 kb *ams* region of the chromosome (Bugert and Geider, 1995) and to induce a hypersensitive response (HR) on non-host plants, encoded by the 30 kb *hrp* region (Kim and Beer, 2000). The large number of *hrp* genes is associated with regulation and transport of two elicitor proteins, HrpN (harpin) (Wei *et al.*, 1992) and HrpW (Barny, 1995). The adjacent *dsp* region with *dspA/E* (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998) may contribute to harpin activity. Because mutagenesis of *hrpN* revealed residual HR-inducing activity of HrpN-fragments, HrpN might not be strictly required as an intact protein (Barny, 1995) and conservation of its sequence has not been strongly selected in mutational changes during evolution. Accordingly, its DNA and amino acid sequences could be open to changes without affecting bacterial fitness and may be useful for strain and species differentiation.

Another molecular tool for differentiation of *E. amylovora* and *E. pyrifoliae* as well as the *Erwinia* strains from Japan is PFGE analysis (Zhang and Geider, 1997; Zhang *et al.*, 1998; Jock *et al.*, 2002). Macrorestriction of the bacterial genome revealed several closely related but distinguishable pattern types for *E. amylovora* which were used to follow spread of fire blight in Europe and in the Mediterranean region (Jock *et al.*, 2002). Another method to distinguish *E. amylovora* strains and the *Erwinia* strains

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from Japan was determination of short sequence DNA repeats (SSR) in the PCR fragment amplified with primers P29A and P29B from the common *E. amylovora* plasmid pEA29 (Kim and Geider, 1999), also applied to the *Erwinia* strains from Japan (Jock *et al.*, 2003a). In contrast to *E. amylovora* strains from Europe and the Mediterranean region, heterogeneous PFGE patterns of American strains could indicate a long persistence of the pathogen in North America. Based on HrpN-sequences, *E. pyrifoliae* strains from Korea (Kim *et al.*, 2001b) and pear-pathogenic *Erwinia* strains from Japan (Kim *et al.*, 2001a) were also divergent. Accordingly, macrorestriction and *hrpN* sequence analysis can be used for differentiation and grouping of strains within the three pathogens.

Results

PFGE patterns of E. amylovora *strains isolated in North America from fruit trees and raspberry*

To estimate possible diversity of *Erwinia amylovora* strains in North America, we collected a set of strains in several areas of Eastern Canada. The samples were derived from fire blight-infected orchards with pear and apple trees located in Nova Scotia near Kentville and in Ontario near Toronto as well as from hawthorn adjacent to the apple orchard in the Kentville area. After an *Xba*I digest (Fig. 1A, Table 1), the strains isolated from hawthorn and apple trees from Kentville carry the PFGE pattern Pt4 as found before (Jock *et al.*, 2002) for strains isolated in England, Western France and Northern Spain. Strikingly, the strains from pears which are isolated in Nova Scotia in an orchard only 100 km apart from the apple orchard, had a different pattern. Another divergent pattern type was found for strains isolated in pear orchards of the Ontario region. The divergence or similarity of the investigated isolates can be deduced from the dendrogram in Fig. 1B.

An additional set of strains was isolated in Eastern Canada 1997 in the Kentville area of Nova Scotia. Strains from apple trees had the same pattern as the strains from hawthorn and apple isolated in 2000 (Table 1). Some shared the PFGE pattern with the European pattern types Pt1, others with Pt4. Most others were quite divergent in contrast to the closely related European pattern types.

Remarkably, *E. amylovora* strains isolated in Europe and in the Mediterranean region have an identical PFGE pattern in an *Spe*I digest except for one band shifted for strains of the *Xba*I pattern type Pt3 (Zhang and Geider, 1997). In contrast, the strains from America were divergent in their *Spe*I pattern (Fig. 2A), except strains EaCa4/97 and EaCa6/97 with an identical *Spe*I pattern, which were isolated in the same year and area. Three strains which were isolated in Eastern Canada from raspberry, an alternative host for fire blight, differed in their PFGE

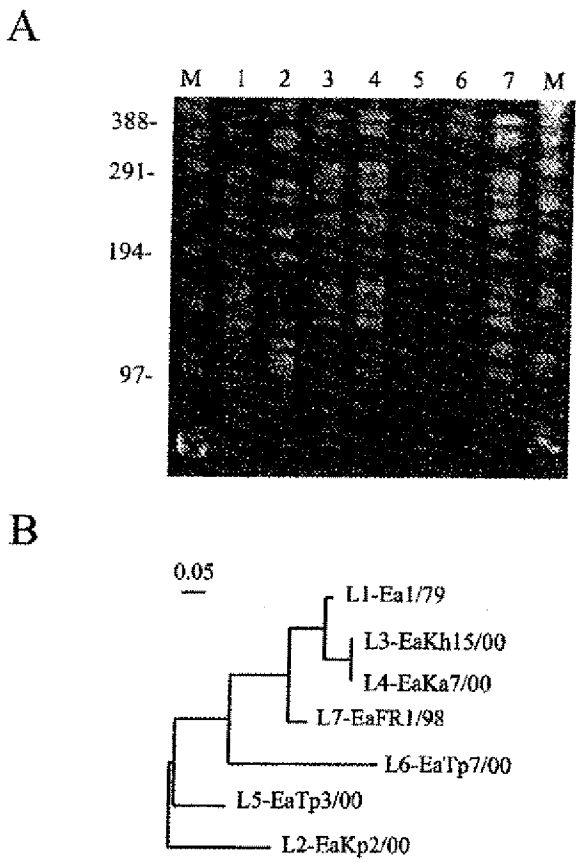

Fig. 1. PFGE analysis of *E. amylovora* strains isolated in Canada after genomic *Xba*I digests.
A. Lanes: M: λ DNA marker (sizes at left in kb); 1: Ea1/79 (Pt1, standard pattern for central Europe); 2: EaKp2/00; 3:EaKh15/00; 4: EaKa7/00; 5: EaTp3/00; 6: EaTp7/00 (isolates from Eastern Canada.); 7: EaFR1/98 (from Germany); *Xba*I digests.
B. Dendrogram from patterns in A. Bar, distance scale.

patterns after *Xba*I and *Spe*I digests among each other and showed barely overlapping patterns with 'fruit tree' strains (Fig. 2A, Table 1). The raspberry strain IL6 from Illinois is more related to the 'fruit tree' strain Ea1/79 than the other rubus strains assayed.

The sizes of SSRs of strains from a narrow region of Eastern Canada

A more variable feature than PFGE patterns of the *E. amylovora* genome is a DNA fragment from the common plasmid pEA29 with several short sequence DNA repeats (Kim and Geider, 1999; Jock *et al.*, 2003a). The SSR numbers are not related to the PFGE patterns, enabling differentiation of strains with the same pattern by SSR numbers. Rarely, the SSR numbers differ for strains isolated from plants in the same region. Nevertheless, strains

Table 1. Bacteria used in the experiments.

Strain	Description of isolation (plant, place, year, provider)	PFGE pattern[a]
E. amylovora strains from Canada (fruit tree)		
EaCa1/00	pear (*P. communis*), Annapolis Valley/Nova Scotia, 2000, G. Braun	A (d)
EaCa4/97	apple (*M. domestica*), Annapolis Valley/Nova Scotia, 1997, G. Braun	B/Pt4 (a)
EaCa6/97	apple, Annapolis Valley/Nova Scotia, 1997, G. Braun	B/Pt4 (a)
EaCaH6	Harrow, D. Hunter	B/Pt4
EaCaH9	Harrow, D. Hunter	Pt1
EaCaL4	London, D. Hunter	B/Pt4
EaCaS16	Simcoe, D. Hunter	Pt1
EaCaS23	Simcoe, D. Hunter	Pt1
EaCaS5	Simcoe, D. Hunter	Pt1
EaCaV15	Niagara, D. Hunter	By
EaCaV18	Niagara, D. Hunter	Bz
EaCaV6	Niagara, D. Hunter	Bx
EaCaW2E	Wentowth country/Hamilton, D. Hunter	Pt1
EaCaW3	Wentowth country/Hamilton, D. Hunter	B/Pt4
EaKa6/00	apple (*M. domestica*), Kentville, this work	B/Pt4
EaKa7/00	apple, Kentville/Nova Scotia, 2000, this work	B/Pt4
EaKa8/00	apple, Kentville/Nova Scotia, 2000, this work	–
EaKa9/00	apple, Kentville/Nova Scotia, 2000, this work	–
EaKa10/00	apple, Kentville/Nova Scotia, 2000, this work	–
EaKh14/00	hawthorn (*Crataegus* sp.), Kentville/Nova Scotia, 2000, this work	–
EaKh15/00	hawthorn (*Crataegus* sp.), Kentville/Nova Scotia, 2000, this work	B/Pt4
EaKh17/00	hawthorn, Kentville/Nova Scotia, 2000, this work	B/Pt4
EaKp1/00	pear (*P. communis*), Kentville/Nova Scotia, 2000, this work	A
EaKp2/00	pear (*P. communis*), Kentville/Nova Scotia, 2000, this work	A
EaKp5/00	pear (*P. communis*), Kentville/Nova Scotia, 2000, this work	–
EaTp3/00	pear (*P. communis*), Niagara Falls/Ontario, 2000, this work	C
EaTp7/00	pear (*P. communis*), Niagara Falls/Ontario, 2000, this work	D
EaTp9/00	pear (*P. communis*), Niagara Falls/Ontario, 2000, this work	–
EaTp10/00	pear (*P. communis*), Niagara Falls/Ontario, 2000, this work	–
EaTp12/00	pear (*P. communis*), Niagara Falls/Ontario, 2000, this work	–
EaTpyr6/00	Asian pear (*P. pyrifolia*), Niagara Falls/Ontario, 2000, this work	–
E. amylovora strains from USA (fruit tree)		
CA1R	apple, California, A. Jones	E
CA263	apple or pear, California, A. Jones	E
CA3R	apple, California, A. Jones	F
Ea88	pear, Washington, A. Jones	E
Ea110	apple, Michigan, A. Jones	B/Pt4
Ea153	USA, L. Pusey	Pt1
EaU8/96	apple, Utah, 1996 (Bereswill *et al.*, 1998)	–
EL01	A. Jones	B/Pt4
FB93-5	pear, Idaho, A. Jones	E
IH3-1	Indian hawthorn, Louisiana, A. Jones	G
IL1196	pear, Washington, A. Jones	E
LA029	pear, Washington, A. Jones	E
LA033	pear, Washington, A. Jones	E
LP100	apple, Washington, A. Jones	E
OR1	pear, Oregon, A. Jones	E
OR6	pear, Oregon, A. Jones	E
UTRJ2	apple, Utah, A. Jones	B/Pt4
WSDA14	apple, Washington, A. Jones	B/Pt4
WSDA34	apple, Washington, A. Jones	E
E. amylovora strains from raspberry (isolated in North America)		
EaCa1/95	raspberry (*Rubus ideus*), Annapolis Valley, Nova Scotia	– (b)
EaCa1/98	raspberry, Bouctouche, New Brunswick	– (bx)
EaCa8/96	raspberry, Bouctouche, New Brunswick	– (c)
EaMR1	raspberry, Michigan	K
EaRKK3	raspberry, Michigan	J
EaRUB7	raspberry (Bereswill *et al.*, 1998)	I
IL6	raspberry, Illinois	H (e)
E. amylovora strains from Europe (Jock *et al.*, 2002)		
CFBP1430	*Crataegus* sp., France, J.-P. Paulin	Pt3a
Ea1/79	*Cotoneaster* sp., Germany, 1979	Pt1 (a)
Ea9–7	*P. communis*, Toulouse (France), 1994	Pt4
Ea296	*C. salicifolius*, Austria, 1993, M. Keck	Pt1
Ea321	CFBP1367, *Crataegus* sp., France, via S. Beer	Pt3
EaFR3/98	*Cotoneaster*, sp., Freiburg (Germany)	Pt1s

Table 1. *Cont.*

Strain	Description of isolation (plant, place, year, provider)	PFGE pattern[a]
EaUK2/98	hawthorn, Kent (UK), 1998	Pt1
P1573	*Cotoneaster* sp., Dorset (UK), 1995, A. Aspin	Pt4
E. pyrifoliae strains from Korea (Kim *et al.*, 2001b)		
Ep1/96	Asian pear (*Pyrus pyrifolia*), South Korea, 1996	PtA
Ep4/97	Asian pear (*P. pyrifolia*) South Korea, 1996	PtB
Ep28/96	Asian pear (*P. pyrifolia*), South Korea, 1996	PtC
Ep31/96	Asian pear (*P. pyrifolia*), South Korea, 1996	PtC
Ep102/98	Asian pear (*P. pyrifolia*), South Korea, 1998	PtA
Erwinia strains from Japan (Kim *et al.*, 2001a)		
Ejp546	Asian pear (*P. pyrifolia*), Hokkaido, 1979, A. Tanii	other
Ejp547[b]	Asian pear (*P. pyrifolia*), Hokkaido, 1979, A. Tanii	PtJp1
Ejp556	Asian pear (*P. pyrifolia*), Hokkaido, 1994, A. Tanii	other
Ejp557	Asian pear (*P. pyrifolia*), Hokkaido, 1994, A. Tanii	PtJp1
Ejp562	Asian pear (*P. pyrifolia*), Hokkaido, 1994, A. Tanii	PtJp1
Ejp617	Asian pear (*P. pyrifolia*), Hokkaido, 1996, R. Roberts	other

a. Letters A to K refer to the pattern of *Xba*I digests, as for Pt1 to Pt4 and PtJp1; highly related pattern are listed with *1*, similar patterns with a lower case letter added to the main type in upper case. (a to e) in this column refer to *Spe*I-digests of genomic DNA as for PtA, PtB and PtC of *E. pyrifoliae*. -, not assayed.
b. Previously named Ejp546a, derived from a culture obtained with Ejp546.

from a narrow area in Nova Scotia were not identical in SSRs displaying numbers of 5, 7, or 9 (Fig. 3, Table 2). These data suggest independent changes of *E. amylovora* populations for SSR. In particular, a strain (EaTp12/00) isolated from a pear tree in the neighbourhood of the orchard, where other strains listed in Table 2, had been isolated, showed a divergent SSR number.

Table 2. SSR numbers of *E. amylovora* strains isolated 2000 in Eastern Canada.

Origin	Isolated from	Name	SSR
Kentville	pear	EaKp1/00	7
		EaKp2/00	7
		EaKp5/00	7
	apple	EaKa6/00	9
		EaKa7/00	9
		EaKa8/00	8
		EaKa9/00	7
		EaKa10/00	5
	hawthorn	EaKh14/00	>10
		EaKh15/00	8
		EaKh17/00	8
Toronto	*P. pyrifolia*	EaTpyr6/00	4
	pear	EaTp9/00	4
		EaTp10/00	4
	pear[a]	EaTp12/00	3

a. From tree adjacent to main orchard.

Sequence analysis of the hrpN *genes of* E. amylovora *'fruit tree' and raspberry strains*

The *hrpN* genes from several *E. amylovora* 'fruit tree' strains with divergent PFGE patterns and from three raspberry strains were cloned by PCR amplification. The European 'fruit tree' strains Ea1/79, CFBP1430, Ea321 (nucleotide sequence from data library), Ea9–3, P1573 or EaFR3/97 with pattern Pt1, Pt3 (2x), Pt4 or Pt1A, respectively, showed almost identical nucleotide sequences for their *hrpN* genes with differences of not more than one nucleotide. On the other hand, the American raspberry strains could be distinguished by their HrpN sequences from 'fruit tree' strains from North America. Three motifs in the N-terminal part are typical for rubus strains and can even be considered diagnostic for their distinction from 'fruit tree' strains (Fig. 3A, boxes). In addition, the rubus strain EaCA1/95 showed a six amino acid insertion sequence in the centre of HrpN and a smaller insertion closer to the N-terminus. These sequences distinguished strain EaCA1/95 from strains EaMR1 and IL6 (Fig. 3A, underlined). In a dendrogram, the 'fruit tree' strain Ea1/79 from Germany is well separated from the aligned American rubus strains, but all *E. amylovora* strains differ in their alignment patterns from the Asian pear pathogens (Fig. 3B).

Sequence analysis of the hrpN *genes of* E. pyrifoliae *strains and Erwinia strains from Japan*

Erwinia amylovora 'fruit tree' and raspberry strains share motifs of HrpN with the Asian pear pathogens. In Fig. 3A, the sequences of the Korean *Erwinia pyrifoliae* Ep1/96 and of an *Erwinia* strain from Japan, Ejp557, were aligned for their possible relationship to the *E. amylovora* raspberry strains. *Erwinia pyrifoliae* strains and the *Erwinia* strains from Japan were strikingly distinct from both *E.*

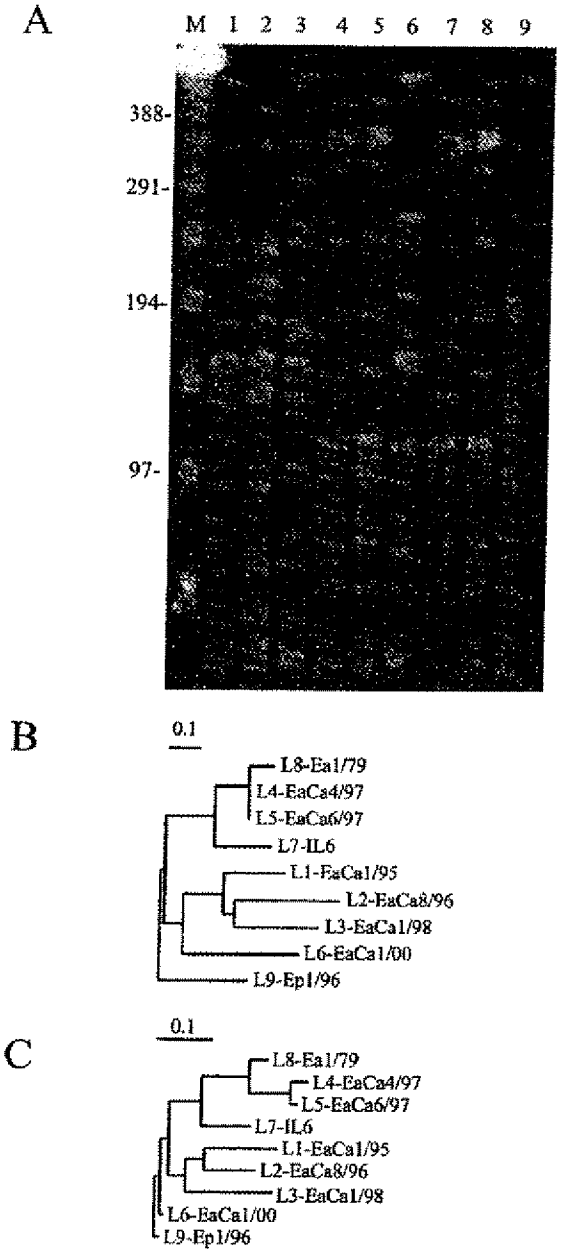

Fig. 2. PFGE analysis of *E. amylovora* strains isolated from raspberry in Canada and Illinois by genomic *Spe*I digests in comparison with isolates from apple in Canada and an *E. amylovora* isolate from cotoneaster and an *E. pyrifoliae* strain.
A. Lanes: M: λ DNA marker (sizes at left in kb); 1: EaCa1/95 (rb); 2: EaCa8/96 (rb); 3: EaCa1/98 (rb); 4: EaCa4/97 (a); 5: EaCa6/97 (a); 6: EaCa1/00 (p); 7: IL6 (rb); 8: Ea1/79 (highest band from partial digest); 9: Ep1/96 (*E. pyrifoliae*).
B. Dendrogram from pattern in A.
C. Dendrogram from pattern of *Xba*I digest with the strains applied in A. Suffix 'a', isolated from apple; 'p', from pear; 'IL6', from raspberry. Bars, distance scales.

amylovora groups. The HrpN sequences of the two Asian pear pathogens were related to each other, but not identical and differed in at least four clusters of more than two amino acids.

The *E. pyrifoliae* strains Ep1/96 and Ep102/98 belong to the PFGE pattern type PtA, Ep4/97 to PtB and Ep28/96, Ep31/96 to pattern type PtC (Kim *et al.*, 2001b). Most parts of their HrpN sequences were identical. Nevertheless, Ep1/96, Ep4/97 and Ep102/98 showed a DNA insertion encoding seven amino acids, which distinguished them from the others (Fig. 4). The motif 'GGSGGGL' is reiterated twice for these strains, but is not conserved for *E. amylovora* or the *Erwinia* strains from Japan (Fig. 3A and Fig. 4A). The distance scale in the dendrogram derived in Fig. 4B indicates a close relationship of the investigated *E. pyrifoliae* strains with small differences. Ep1/96, Ep28/96 and Ep102/98 are highly related, less Ep31/96, whereas Ep4/97 is more distinct from the others.

The *Erwinia* strains from Japan analysed were also not completely homogenous for their HrpN sequences. Strains Ejp547, Ejp557 and Ejp562 were highly related in the PFGE patterns after *Xba*I digests, whereas the others could be separated from the first group on this basis (Kim *et al.*, 2001a). In agreement with those data, the HrpN proteins of Ejp547, Ejp557 and Ejp562 differed from the amino acid sequences derived from the other strains at five sites of HrpN (Fig. 5A). The dendrogram in Fig. 5B indicates the relationship of Ejp557, Ejp547 and Ejp562, separating them from the other strains, also confirming PFGE data that strain Ejp547 is not identical with strain Ejp546, obtained in the same agar culture.

The sequences of the *hrpN* genes of *E. amylovora* 'fruit tree' and raspberry strains as well as of *E. pyrifoliae* strains and *Erwinia* strains from Japan showed a differential degree of conservation. The *E. amylovora* 'fruit tree' and rubus strains were 97% related to each other, whereas the HrpN proteins of *E. pyrifoliae* and *Erwinia* strains from Japan had only 83% similarity to HrpN of *E. amylovora* 'fruit tree' strains.

A summarizing dendrogram (Fig. 6) grouped the *E. amylovora* strains apart from the two Asian pear pathogens. *E. pyrifoliae* strains are highly related to each other, and less to the *Erwinia* strains from Japan.

Discussion

The PFGE patterns of the strains isolated in North America are divergent, in contrast to the pattern of strains from Central Europe and the Mediterranean region, which were grouped into four main pattern types (Jock *et al.*, 2002). In spite of basically unrestricted trade in fruit and fire blight host plants, there has been no obvious mixing of pattern types in Europe and the Mediterranean region. Sequential spread from infected sites is the dominant way of disease

A

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Ea1/79     (1)  mslntsglgastmqisiggaggnnglltgtsrqnaglggnsalglgggnqndtvnqlaglltgmmmmmsmmggggl---mg
IL6        (1)  ..........................................................................---..
EaMR1      (1)  ..........................................................................---..
EaCa1/95   (1)  ..........................................................................---..
Ep1/96     (1)  ......a..............g..............dh.........n.........m.................tgll.
Ejp557     (1)  ......a..............g..............dh.........n.........m.................---t.

Ea1/79    (78)  ----gglgggl-----gngl--g-gsgg-----------lgeglsnalndmlggsln-----tlgskggnnttsttnsp
IL6       (78)  lmg.....-----.-------.-----------.....f...g-----.....
EaMR1     (78)  lmg.....-----.-------.-----------.....f...g-----.....
EaCa1/95  (78)  lmg.....-----.g..-.n.l.-----------.....f...g-----.....
Ep1/96    (81)  ----..f....lgggs.g..-.-.....glgglgghlgst..g.igggigga...p.gatvgts...gi.gsaa.gvg.a
Ejp557    (78)  llgg......f-----.g..lg.-.....glgglggdlgst..g..gagig.a...p.gatvgts...gi.gsaa.gvg.a

Ea1/79   (129)  ldqalginstsqnd------dst------sgtdstsdssdpmqqllkmfseimqslfgdgqdgtqgssggkqptegeqn
IL6      (129)  ..............------..------.....................................................
EaMR1    (129)  ..............------..------.....................t...............................
EaCa1/95 (133)  ..............-------...fgtdst.....................................................
Ep1/96   (154)  ..............------s..------.....s.....v...m.............e......sg..a.........s
Ejp557   (152)  ..............sstsgt..-------.....s.....v...m.............e......sg..a.........s

Ea1/79   (197)  aykkgvtdalsglmgnglsqllgnggllgggqggnagtgldgsalggkglqnlsgpvdyqqlgnavgtgigmkagiqalnd
IL6      (197)  .....................................................................................
EaMR1    (197)  .....................................................................................
EaCa1/95 (207)  ..................f..................................................................
Ep1/96   (222)  ......s....a........t............s........g.....................................
Ejp557   (226)  ......s....a........t.......a....s........g.....................................

Ea1/79   (277)  igthadsstrsfvnkgdramakeigqfmdqypevfgkpqyqkgpgqevkttddkswakalskpdddgmtpasmeqfnkakg
IL6      (277)  .....................................................................................
EaMR1    (277)  .....................................................................................
EaCa1/95 (287)  .............d.......................................................................
Ep1/96   (302)  .....................................................................................
Ejp557   (306)  .....................................................................................

Ea1/79   (357)  miksamagdtgngnlqargaggsalgidammagdainnmalgk
IL6      (357)  ................h.....................
EaMR1    (357)  ........................................
EaCa1/95 (367)  ........................................
Ep1/96   (382)  ..................................t.....
Ejp557   (386)  ..................................t.....
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B

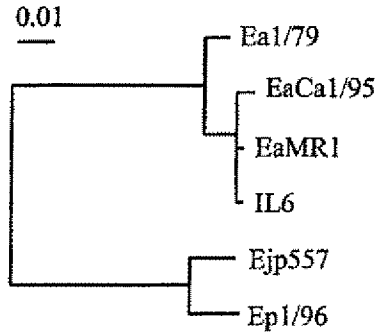

Fig. 3. Sequence alignment of the HrpN proteins from raspberry strains isolated in Canada compared with the *E. amylovora* 'fruit tree strain' Ea1/79, *E. pyrifoliae* Ep1/96 and Ejp557, an *Erwinia* strain from Japan.
A. Common motifs for raspberry strains are boxed and unique insertions for strains are underlined.
B. Dendrogram from the amino acid sequences aligned in A. Bar, distance scale.

A

```
Ep1/96     (1)  mslntsalgastmqisiggagggnglltgtsrqnaglgdhsalglgggnnndtvnqlagmltgmmmmmsmsmgggltgllg
Ep4/97     (1)  ...............................................................................
Ep28/96    (1)  ...............................................................................
Ep31/96    (1)  ...............................................................................
Ep102/98   (1)  ...............................................................................

Ep1/96     (81) ggfgggllggsgggglggsggglgglgghlgatlgggigggiggalggplgatvgtslgsgiggsaasgvgsaldqalgi
Ep4/97     (81) ..................s........d.....................................................
Ep28/96    (81) ..........------.....d.....................................................
Ep31/96    (81) ..........------.....v...d.....................................................
Ep102/98   (81) ..........-----.....d.....................................................

Ep1/96     (161) nstsqndsstsgtdsssdssdpvqqlmkmfseimqslfgegqdgtqsgssagkqptegeqsaykkgvsdalsalmgngls
Ep4/97     (161) ...............................................................................
Ep28/96    (154) ...............................................................................
Ep31/96    (154) ...............................................................................
Ep102/98   (161) ...............................................................................

Ep1/96     (241) qtlgngglgggggsagtgldgsglggkglqnlsgpvdyqqlgnavgtgigmkagiqalndigthsdsstrsfvnkgdra
Ep4/97     (241) ...............................................................................
Ep28/96    (234) ...............................................................................
Ep31/96    (234) ...............................................................................
Ep102/98   (241) ...............................................................................

Ep1/96     (321) makeigqfmdqypevfgkpqyqkgpgqevktddkswakalskpdddgmtpasmeqfnkakgmiksamagdtgngnlqarg
Ep4/97     (321) ..........................................e....................................
Ep28/96    (314) ...............................................................................
Ep31/96    (314) ...............................................................................
Ep102/98   (321) ...............................................................................

Ep1/96     (401) aggsslgidammagdtinnmalgk
Ep4/97     (401) ........................
Ep28/96    (394) ........................
Ep31/96    (394) ...........r............
Ep102/98   (401) ........................
```

B

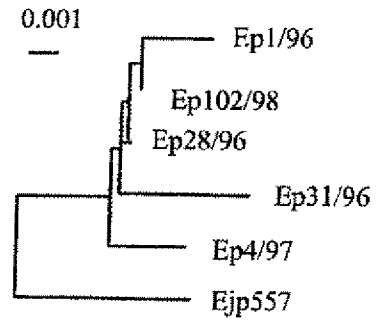

Fig. 4. Comparison of HrpN proteins from five *E. pyrifolia* strains.
A. Amino acid aligment. The motif for strain differentiation is boxed.
B. Dendrogram from the amino acid sequences aligned in A. Bar, distance scale.

distribution, except for introduction of fire blight into Central Spain and Northern Italy, where plant imports can be connected with appearance of fire blight caused by *E. amylovora* strains displaying pattern type Pt3, which has not been found in the adjacent regions.

An ordered PFGE pattern was not found for strains from North America, because even a relatively low number of isolates gave rise to several different patterns. They also differ from European patterns except for Pt1 and Pt4, which were found repeatedly in isolates from Eastern Canada. Thus, Pt1 and Pt4 could have originated in North America and were then distributed to Europe (Jock *et al.*, 2002), first to England with the first European fire blight outbreaks (Billing and Berrie, 2002). The other patterns in

A

```
Ejp546   (1) mslntsalgastmqisiggagggngllgtsrqnaglgdhsalglgggnnndtvnqlagmltgmmmmmmmmgggggltgllg
Ejp547   (1) ................................................................................
Ejp556   (1) ................................................................................
Ejp557   (1) ................................................................................
Ejp562   (1) ................................................................................
Ejp617   (1) ................................................................................

Ejp546  (81) ggglgggfgggllgggsgggglgglggdlgstlggglgagiggalggplgatvgnslgssiggsaasgagsaldqalgins
Ejp547  (81) ..............................................t....g.......v.............
Ejp556  (81) ................................................................................
Ejp557  (81) ..........................................d.........t....g.......v.............
Ejp562  (81) ..............................................t....g.......v.............
Ejp617  (81) ................................................................................

Ejp546 (161) tsqndsstsgtds------esdsedpvqqlmkmfseimqslfgegqdgtqsgesagkqptegeqsaykkgvsdalsalmg
Ejp547 (161) ............tsgtds............................................................
Ejp556 (161) ............------............................................................
Ejp557 (161) ............tsgtds............................................................
Ejp562 (161) ............tsgtds............................................................
Ejp617 (161) ............------............................................................

Ejp546 (235) nglsqtlgngglgggqggsagtgldgsglggkglqnlsgpvdfqqlgnavgtgigmkagiqalndigthsdsstrsfvnk
Ejp547  (241 ........................................y.............................n......
Ejp556 (235) ................................................................................
Ejp557 (241) ............a.......................................y.....................
Ejp562 (241) ........................................y.....................................
Ejp617 (235) ................s...................................................................

Ejp546 (315) gdramakeigqfmdqypevfgepqyqkgpgqevktddkswakalskpdddgmtpaameqfnkakgmiksamagdtgngnl
Ejp547 (321) .ep..................k...............................................................
Ejp556 (315) ....................k...............................................................
Ejp557 (321) ....................k...............................................................
Ejp562 (321) ....................k...............................................................
Ejp617 (315) ....................k.......k.......................................................

Ejp546 (395) qargaggsslgidammagdtinnmalgk
Ejp547 (401) ............................
Ejp556 (395) ............................
Ejp557 (401) ............................
Ejp562 (401) ............................
Ejp617 (395) ............................
```

B

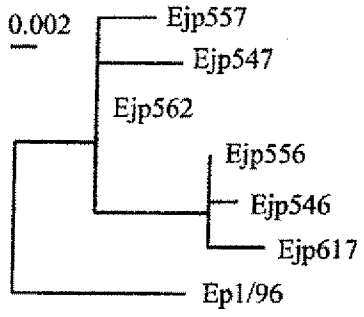

Fig. 5. Comparison of HrpN proteins from six *Erwinia* strains isolated in Japan.
A. Amino acid alignment. An insertion motif and amino acid substitutions for strains Ejp547, Ejp557, and Ejp562 are underlined.
B. Dendrogram from the amino acid sequences aligned in A. Bar, distance scale.

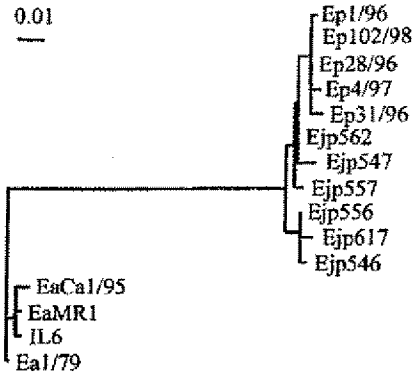

Fig. 6. A dendrogram showing the relatedness of *E. amylovora* 'fruit tree' and raspberry strains and the evolutionary distance of the Asian pear pathogens based on the HrpN amino acid sequences. Bar, distance scale.

America can be explained by genetic changes over a long time period; they were rarely distributed to other countries except for Pt2, a pattern which was found first in Egypt and also in a strain from California (Zhang and Geider, 1997; Jock *et al.*, 2002).

A special subgroup of *E. amylovora* strains from raspberry is endemic in North America and has only been isolated there. A reason for the difference in the PFGE patterns from *E. amylovora* 'fruit tree' strains could be the unusual host, which required many genomic changes for adaptation of the pathogen. On the other hand, their presumably long persistence in North America could have allowed accumulation of many base changes in the genome causing their pattern heterogeneity.

The SSR numbers are not related to PFGE patterns or the areas of isolation as also found for American (Schnabel and Jones, 1998) and European *E. amylovora* strains (Kim and Geider, 1999). Nevertheless, different numbers indicate non-identical isolates from fire blighted plants. Among intermediate numbers there is a high occurrence of low numbers such as 3 and 4, which are not often observed in Central Europe. In isolations from the same apple orchard in Kentville, we found SSR numbers from 5, 7 and 9. Normally, only one SSR-type is usually isolated in the same set of isolates, but recently, we observed some exceptions like in England where we found SSR type 3 and 7 in isolates from adjacent plants (Jock *et al.*, 2003a).

The ability to induce a hypersensitive response (HR) on non-host plants is a common feature of plant pathogenic bacteria. In evolution, many genes of the *hrp* cluster especially those involved in protein secretion have been highly conserved among bacteria (Van Gijsegem *et al.*, 1993; Bogdanove *et al.*, 1996). A spontaneous base change in *hrpL* within an *E. pyrifoliae* population has been recently described (Jock *et al.*, 2003b). Genes encoding harpins are highly divergent even for related bacteria. The HrpN proteins of two related species such as *P. stewartii* ssp. *stewartii* (*E. stewartii*) and *P. stewartii* pv. gypsophylae show only 60% similarity to each other (EMBL Nucleotide Sequence Database accession numbers AF282857 and AF21176 respectively). The similarity of these harpins and HrpN of *E. carotovora* ssp. *carotovora* (AF302656) to harpin of *E. amylovora* is 62%, 56%, and 49% respectively. The sequence information of *hrpN* is not only suited for classification of bacterial species, but also for grouping of strains within a species. On the other hand, HrpN can be conserved, found for *E. amylovora* 'fruit tree' strains, where the sequences matched at the nucleotide level. These strains isolated from raspberry in North America, share more than 95% similarity. A high relationship was also observed between *E. pyrifoliae* strains from Korea and the Japanese pear pathogen, whereas *E. amylovora* strains match with these pathogens less than 85%. Although the *Erwinia* stains from Japan have not been taxonomically classified, the relatedness of HrpN proteins adds to the notion to place these with *E. pyrifoliae* into the same species (Kim *et al.*, 2001a). In addition, HrpN sequences provided also information for strain differentiation within a species.

Because the transport of harpin depends on several cellular proteins, its sequence cannot freely change only to conserve its elicitor activity. Whether the HrpN protein or even the DspA/E-protein (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998) contribute to host plant specificity of a pathogen has still to be shown. The divergences of the HrpN sequences should indicate an evolutionary drift, similar to the PFGE patterns analysed. The most likely explanation is the long persistence of *E. amylovora* in North America, of *E. pyrifoliae* in Korea and the slightly different pear pathogen in Japan. Furthermore, the occurrence of European pattern types Pt1 and Pt4 among the divergent American PFGE patterns may indicate a rare escape of fire blight from its origin in North America.

Experimental procedures

Bacterial strains, PCR and PFGE analyses

The *E. amylovora* strains were isolated in the Eastern part of Canada, or were gifts from colleagues (Table 1). They were confirmed as *E. amylovora* on several agar plates including MM2Cu (Bereswill *et al.*, 1998) and by PCR assays (Bereswill *et al.*, 1992). Pulsed-field gel electrophoresis analysis (Jock *et al.*, 2002) and determination of the SSR numbers (Kim *et al.*, 1999) were done as described. *Erwinia pyrifoliae* (Kim *et al.*, 2001b) and the *Erwinia* strains from Japan were also described previously (Kim *et al.*, 2001a). For pattern comparison, the PFGE fragments were assigned by eye with letters and the program CLUSTALX1.81 used for pairwise alignments. The dendrograms were adjusted with NJ-tree and further processed in a graphics program. Pattern analy-

sis was also done with the public domain programs ImageJ (v. 1.30; W. Rasband, NIH, USA) and Cross Checker (v. 2.91; J. B. Buntjer, Wageningen, the Netherlands) and alignment with Treecon vs. 1.3b (Y. van de Peer, Konstanz, Germany) and CLUSTALX1.81 respectively. Corrections by eye were required for further adjustment of the band assignments.

Analysis of the hrpN *genes from* E. amylovora *and the Asian pear pathogens*

The *hrpN* genes of strains from the three pathogens were amplified with PCR consensus primers, which were deduced by comparison of several known nucleotide sequences from plant pathogens namely *E. amylovora* (EMBL Nucleotide Sequence Database accession number M92994) or *P. stewartii* (accession number AF282857). Primer HRPN1 was 5′-ATGAGTCTGAATACAAG-3′ (at start of *E. amylovora hrpN*) and primer HRPN3c 5′-GCTTGCCAAGTGCCATA-3′ (in *hrpN*, 11 bp downstream from stop codon). In some cases, weak PCR bands obtained could indicate incomplete matching of the primers. The amplified DNA fragments were cloned into vector pGEM-T and were commercially sequenced. To cover the total *hrpN* genes, a third primer HRPMc (5′-CCACGGCGTTACCCAACTGCTGG-3′) located in the central part of the *hrpN* gene was used to cover gaps in the HrpN sequences. Alignments and dendrograms were created with CLUSTALX1.81.

Erwinia pyrifoliae and the *Erwinia* strains from Japan were considered to be sufficiently related to *E. amylovora* to amplify their *hrpN* genes with the *Erwinia* PCR consensus primers given above. This was indeed possible and allowed cloning and sequencing of their *hrpN* DNA fragments as for *E. amylovora* by using primers HRPN1 and HrpN3c. A primer comprising the stop codon at the C-terminus of *hrpN* did not result in the formation of a PCR product together with primer HRPN1.

The *hrpN* nucleotide sequences from strains Ea1/79, EaCa1/95, IL6, EaMR1, Ejp546, Ejp557, Ep1/96, Ep31/96, Ep4/97 and were deposited in the EMBL Nucleotide Sequence Database with the accession numbers AJ579689 (Ea1/79), AJ579690 (EaCa1/95), AJ579691 (IL6), AJ579692 (EaMR1), AJ579693 (Ejp546), AJ579694 (Ejp557), AJ579695 (Ep1/96), AJ579696 (Ep31/96) and AJ579697 (Ep4/97).

Acknowledgements

Samples of necrotic plants from orchards with fire blight were collected by us in Canada with the help of Gordon Braun, Paul Hildebrand (Kentville/Nova Scotia), Martin Hubbes and David Hunter (Toronto/Ontario). We also thank Al Jones (East Lansing/Michigan), David Hunter and Gordon Braun for sending additional *E. amylovora* strains as well as Carlo Bazzi (Bologna) and Christopher Hayward (Brisbane) for comments on the manuscript.

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MINIREVIEW

The Type III (Hrp) Secretion Pathway of Plant Pathogenic Bacteria: Trafficking Harpins, Avr Proteins, and Death

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INTRODUCTION

The ability of plant pathogenic bacteria to deliver death-triggering proteins to the interior of plant cells was revealed in a rapid succession of papers in 1996 that transformed our concepts of bacterial plant pathogenicity. The breakthrough came with the convergence of work on Hrp systems and Avr proteins, an understanding of which requires an introduction to the most prevalent bacterial pathogens of plants, the cardinal importance of the Hrp pathway, and the paradoxical phenotype associated with *avr* genes.

Plant pathogenic bacteria in the genera *Erwinia*, *Pseudomonas*, *Xanthomonas*, and *Ralstonia* cause diverse, and sometimes devastating, diseases in many different plants, but they all share three characteristics: they colonize the intercellular spaces of plants, they are capable of killing plant cells, and they possess *hrp* genes. Many of these pathogens are host specific. In host plants, they produce various symptoms after several days of multiplication, whereas in nonhost plants, they trigger the hypersensitive response (HR), a rapid, defense-associated, programmed death of plant cells at the site of invasion (21, 43). With inoculum levels typically encountered in natural environments, the HR produces individual dead plant cells that are scattered within successfully defended healthy tissue (71). However, experimental infiltration of high inoculum levels (>10^6 bacterial cells/ml) results in macroscopically observable death of the entire infiltrated tissue, usually within 24 h (42). Pioneer screens for random transposon mutants with impaired plant interactions yielded a prevalent class that was designated Hrp$^-$, that is, deficient in both HR elicitation in nonhost plant species and pathogenicity (and parasitic growth) in host species (49, 56). This complete loss of pathogenic behavior results from mutation of any one of several *hrp* genes, which largely encode components of a type III protein secretion system (73). Because the capacity to elicit the HR is a convenient marker for the capacity to be pathogenic and these two abilities have a common genetic basis, the "simple" problem of HR elicitation is being studied as an entry to the larger problem of pathogenesis.

A key part of the HR puzzle is that HR elicitation and the resulting limitation in host range can occur if the pathogen possesses any one of many possible *avr* (avirulence) genes that interact with corresponding *R* (resistance) genes in the host plant. Such "gene-for-gene" interactions result in recognition of the bacterium and the triggering of plant defenses. For example, *Pseudomonas syringae* pv. glycinea is one of over 40 *P. syringae* pathovars differing largely in host range among plant species and is subdivided into races on the basis of their interactions with genetically distinct cultivars of its host, soybean. Those race-cultivar interactions involving matching bacterial *avr* and plant *R* genes result in the HR and avirulence, i.e., failure of the bacterium to produce disease. The *R* genes encode components of a parasite surveillance system and are crossed into crops from wild relatives by plant breeders for disease control. *avr* genes are identified and cloned on the basis of the avirulence they confer on virulent races in appropriate test plants (39, 69). In most cases, it is not clear why plant pathogens carry *avr* genes that betray them to host defenses but new insights into this question are discussed below.

Both *hrp* and *avr* genes were originally defined on the basis of the phenotypes they confer on bacteria interacting with plants. Molecular studies have revealed a functional relationship between the products of these two classes of genes and an underlying similarity with a key virulence system of several animal pathogens. *Yersinia*, *Salmonella*, and *Shigella* spp. transfer virulence effector proteins directly into animal cells via the type III pathway (16, 17, 62, 67, 84). Similarly, plant pathogens use the Hrp type III pathway to transfer Avr effector proteins to the interior of plant cells. The genetic dissection of type III secretion systems is just beginning, and little is known of the mechanisms of protein translocation. In this review, we will describe (i) the recently completed inventory of genes directing type III secretion in plant pathogens and new insights into type III secretion mechanisms gained from research with Hrp systems, (ii) two classes of proteins (harpins and pilins) that are secreted by the Hrp type III pathway when plant pathogens are grown in media that mimic plant intercellular fluids, (iii) evidence that Avr proteins are delivered by the Hrp pathway directly to the interior of plant cells, and (iv) a resulting new paradigm for bacterial plant pathogenicity. The focus will be on quite recent work, and readers are referred to other reviews for a classic introduction to the HR phenomenon (43), earlier investigations of the Hrp system (11), *avr* genes (20, 46), and a wider perspective on bacterial virulence systems and plant responses (2).

Hrp PROTEIN SECRETION SYSTEM

***hrp* and *hrc* genes.** *hrp* genes have been extensively characterized in four representative gram-negative plant pathogens: *P. syringae* pv. syringae (brown spot of bean), *Erwinia amylovora* (fire blight of apple and pear), *Ralstonia* (*Pseudomonas*) *solanacearum* (bacterial wilt of tomato), and *Xanthomonas campestris* pv. vesicatoria (bacterial spot of pepper and tomato). Most of the known *hrp* genes in these strains are contained in chromosomal clusters of about 25 kb (Fig. 1). In at least some cases, the *hrp* clusters are sufficient to allow HR elicita-

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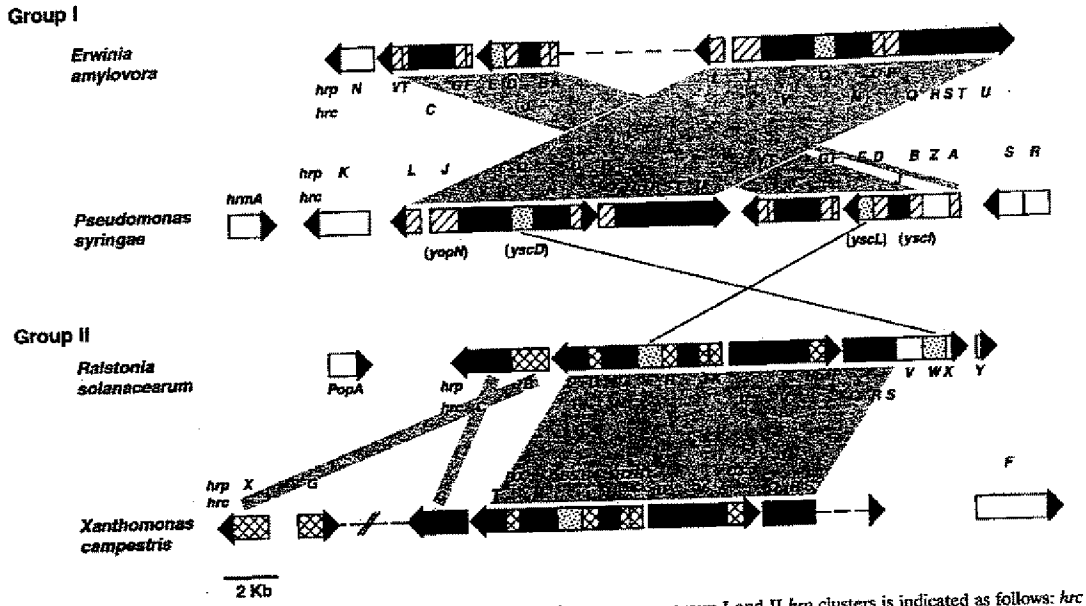

FIG. 1. *hrp* gene clusters of four model plant pathogens. The distribution of each gene among group I and II *hrp* clusters is indicated as follows: *hrc* genes, dark shading; *hrp* genes that are conserved between groups I and II but show weaker similarity to *Yersinia ysc* genes than *hrc* genes, stippling (the two lines between groups indicate homologs); genes common to group I, diagonal lines; genes common to group II, hatching; genes for which no homologs have been reported, white. Dashed lines indicate gaps in the reported sequence of each *hrp* cluster. The shaded bands between members of a group indicate colinear gene arrangements. Note that homologous *hrp* genes have the same designation within group I but not within group II. *Yersinia* genes for which similarity has been noted with *hrp* genes of *R. solanacearum* (74), *E. amylovora* (10, 41), and/or *P. syringae* (36, 60) are in parentheses below the pair of group I *hrp* clusters. The *hrp* cluster of *R. solanacearum* is carried on a megaplasmid (12), but the others appear to be chromosomal. See reference 9 for previous designations of *hrc* genes and for references to all but the recent sequence reports in references 38, 41, and 81.

tion (but not disease) by nonpathogenic bacteria such as *Escherichia coli* and *Pseudomonas fluorescens* (8, 37).

Initial sequencing of the *hrp* clusters from *R. solanacearum*, *X. campestris* pv. vesicatoria, and *P. syringae* pv. syringae revealed homologies with components of the virulence protein (Yop) secretion system of *Yersinia* spp. (22, 29, 34), thereby suggesting the existence of a conserved "type III" protein secretion pathway in gram-negative pathogens of both plants and animals (65, 73). The near completion of these sequences has revealed further homologies and has led to two major changes in the nomenclature of *hrp* genes (9). First, those *hrp* genes that are broadly conserved in pathogenic *Pseudomonas*, *Erwinia*, *Ralstonia*, *Xanthomonas*, *Yersinia*, *Salmonella*, and *Shigella* spp. were redesignated *hrc* (HR and conserved) and given the last-letter designations of their *Yersinia ysc* homologs. The designations for Hrc homologs in various bacteria outside of the plant pathogen group are presented in Table 1. When referred to broadly, the term "*hrp* genes" is intended to encompass the *hrc* subset (9). Second, the *hrp* gene concept was widened to include homologous genes in plant pathogens where mutations do not lead to typical Hrp phenotypes. For example, mutations in *hrp* homologs result in loss of the Wts (watersoaking) phenotype in *Erwinia stewartii* (Stewart's wilt of corn) and reduced infectivity at low inoculum levels in *Erwinia chrysanthemi* (bacterial soft rot) (6, 23). Thus, the *hrp* genes appear to be universal among plant pathogenic *Erwinia*, *Pseudomonas*, *Ralstonia*, and *Xanthomonas* spp. and they control a variety of bacterium-plant interaction phenotypes in addition to the HR.

Group I and II *hrp* clusters. The four *hrp* clusters that have been most characterized can be divided into two groups based on their possession of similar genes, operon structures, and regulatory systems (2). The *hrp* clusters of *P. syringae* and *E. amylovora* are in group I, and those of *R. solanacearum* and *X. campestris* are in group II. In addition to the nine *hrc* genes, two *hrp* genes are conserved between the group I and II *hrp* clusters and show some similarities to *ysc* genes (Fig. 1) (10, 36, 41, 74). It is likely that more of the present *hrp* genes will be discerned as belonging to the *hrc* category with additional data on the structure, function, and conservation of their products in both plant and animal pathogens. Nevertheless, some of the *hrp* genes appear to be completely different between the two groups, the arrangements of genes within some operons are characteristic of each group, and the regulatory systems are distinct (Fig. 1). A key difference in regulation is that group I *hrp* operons are activated by HrpL, a member of the ECF (extracytoplasmic function) subfamily of sigma factors (50, 78, 85), whereas most group II *hrp* operons are activated by a

TABLE 1. Hrc proteins of plant pathogenic bacteria and their animal pathogen and flagellar homologs

Plant pathogen protein[a]	*Yersinia* protein	*Salmonella* protein	*Shigella* protein	Flagellar protein(s)
HrcC	YscC	InvG	MxiD	
HrcJ	YscJ	PrgK	MxiJ	FliF
HrcN	YscN	SpaL	Spa47	FliL
HrcQ	YscQ	SpaO	Spa33	FliN, -Y
HrcR	YscR	SpaP	Spa24	FliP
HrcS	YscS	SpaQ	Spa9	FliQ
HrcT	YscT	SpaR	Spa29	FliR
HrcU	YscU	SpaS	Spa40	FlhB
HrcV	LcrD	InvA	MxiA	FlhA

[a] References for the sequences of *hrc* genes and all homologs are compiled in references 9, 25, and 74.

member of the AraC family, which is designated HrpB in *R. solanacearum* and HrpX in *X. campestris* (27, 57, 82). However, *hrp* genes in both groups are generally repressed in complex media and expressed in plants and in media that mimic plant intercellular fluids (11).

Functions of Hrp and Hrc proteins in type III protein secretion. With the *hrp* clusters of four representative plant pathogens now almost completely sequenced, analysis of the functions of individual components is beginning. Nonpolar mutations have been constructed in most of the *hrp* and *hrc* genes in *R. solanacearum* and in some of the genes in *P. syringae* pv. syringae and *E. amylovora* (10, 15, 54, 77). The results suggest that the secretion apparatus requires all of the *hrc* genes (*hrcQ* awaits testing). The *R. solanacearum* mutant analysis also reveals a requirement for *hrpF*, *-W*, *-K*, and *-X* (54). As discussed above, *hrpF* and *hrpW* have group I and possible *ysc* homologs. Thus, the Hrp type III secretion apparatus is likely composed of a core of 13 proteins, all but 2 of which appear to be broadly conserved. The predicted locations and functions of most of these proteins have been systematically presented for the *R. solanacearum* Hrp system (74), and they appear to be the same in *X. campestris*, *E. amylovora*, and *P. syringae*.

Sequence comparisons reveal that all of the Hrc proteins, other than HrcC, have a homolog involved in flagellum-specific export or early events in flagellum biogenesis (Table 1). The abilities of the presumably more ancient flagellar system to regulate the order (and possibly amount) of protein released and to secrete proteins in association with an extracellular appendage are properties that may be particularly important in the type III transfer of virulence proteins into host cells (18, 52). Plant pathogens offer several experimental advantages for exploring mechanisms of type III secretion and, indirectly, flagellum-specific secretion. The flagellum-specific and animal pathogen type III secretion systems have been difficult to study because many mutations pleiotropically disrupt production of the secretion apparatus and the secreted proteins. For example, the *Yersinia pestis* LcrD and *Bacillus subtilis* FlhA proteins (homologs of HrcV) were initially thought to have primary functions in regulation (14, 59). However, the unambiguous secretion phenotype of an *E. amylovora hrcV* mutant provided strong evidence that the primary function of members of this protein superfamily is in secretion (77). Plant pathogens offer other experimental advantages for exploring type III secretion mechanisms: defined subclones of ca. 25 kb are conveniently sufficient for Hrp-mediated secretion by *E. coli* and other model bacteria (31, 77), and *hrc* gene arrangements and mutant phenotypes suggest that translocation across the inner and outer membranes is partially separable in these bacteria (15).

In both group I and II *hrp* clusters, the six *hrc* genes predicted to encode a flagellum-derived system for Sec-independent translocation across the inner membrane (*hrcN*, *-R*, *-S*, *-T*, *-U*, and *-V*) are in operons other than that containing the one *hrc* gene predicted to direct translocation across the outer membrane (*hrcC*) (Fig. 1 and 2). HrcC is a member of the PulD/pIV superfamily of outer membrane proteins, which are involved in type II protein secretion (PulD) and filamentous phage secretion (pIV) (26). These proteins form homomultimers in the outer membrane which permit phage or protein exit and induce the *psp* (phage shock protein) operon (63). The HrcC protein of *X. campestris* pv. vesicatoria was the first member of the type III branch of this superfamily shown to induce the *psp* operon, thereby suggesting that the type III pathway also employs an outer membrane, channel-forming multimer (80). A *P. syringae* pv. syringae *hrcC* mutant accumulates some of the normally secreted HrpZ harpin (discussed below) in the periplasm, whereas a *hrcU* mutant accumulates

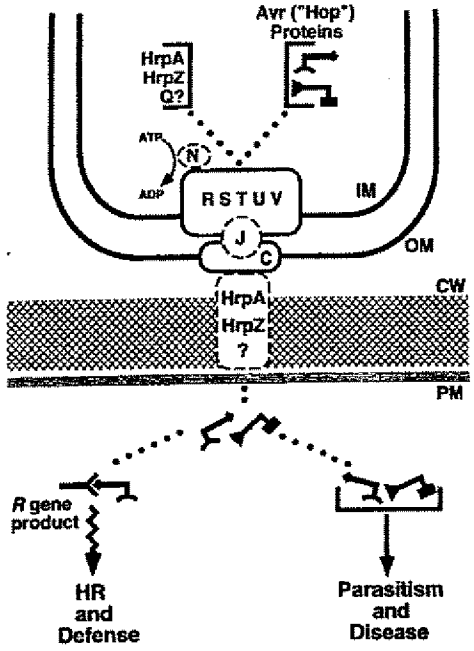

FIG. 2. Model for the delivery of parasite-promoting Avr proteins (i.e., Hop proteins according to a proposal discussed in the text) into plant cells by the Hrp type III secretion system (*P. syringae* example). To reach their targets, Avr proteins must cross the bacterial inner membrane (IM), outer membrane (OM), plant cell wall (CW), and plasma membrane (PM). Only Hrc components (indicated by their last letters in predicted subcellular locations) and proteins known to be secreted are shown. The location of hydrophilic HrcQ ($HrcQ_A$ and $HrcQ_B$ in *P. syringae*) is unknown, but the homologous SpaO is secreted by *Salmonella* spp. (25, 48). Four additional Hrp proteins, not shown, appear to be required for secretion (see text). Dashed-line boxes indicate uncertainties about precise location. For example, it is not known whether HrpA or HrpZ penetrates the plant cell wall and whether these and/or other Hrp proteins trigger Avr transfer into plant cells by endocytosis. Secretion of HrpA and HrpZ is not dependent on plant cell contact, whereas secretion of Avr proteins apparently is. Once inside plant cells, multiple Avr proteins apparently promote parasitism collectively by unknown mechanisms (short arms denote weak phenotypes of virulence domains interacting with undefined host targets), unless any one of the proteins interacts with a host *R* gene product, thereby triggering the HR defense. Mutation of a host target, to diminish benefit to the parasite, and detection by the *R* gene surveillance system are likely evolutionary responses of plants to the bacterial deployment of a new virulence protein; coevolution would be expected to generate many *avr* and *R* genes in complex populations of plants and bacterial parasites.

the protein exclusively in the cytoplasm (15). Thus, the sequence-based prediction that separate inner and outer membrane translocator systems have been recruited to form the Hrp pathway is supported by a novel secretion phenotype revealing partial separation of these functions (15).

HARPINS, PILINS, AND OTHER PROTEINS SECRETED IN CULTURE BY THE Hrp SYSTEM

Harpins. Broadly defined, harpins are glycine-rich proteins that lack cysteine, are secreted in culture when the Hrp system is expressed, and possess heat-stable HR elicitor activity when they infiltrate the leaves of tobacco and several other plants. As is characteristic of proteins secreted by the type III pathway, harpins lack an N-terminal signal peptide. The first harpin was discovered in the culture fluids of *E. coli* cells carrying a highly expressed *hrp* cluster from *E. amylovora* (79). Because mutations in the harpin-encoding *hrpN* gene in *E. amylovora* strongly diminish HR elicitation in tobacco and pathogenicity in susceptible, immature pear fruits, harpin was initially thought to be the primary virulence protein traveling the Hrp pathway

(79). Subsequent analysis of harpins from other bacteria has revealed that harpins differ substantially in their primary structure and their contribution to Hrp phenotypes, and their actual function is unknown (4, 7, 19, 31).

The harpin genes of *E. amylovora* (*hrpN*) (79), *E. chrysanthemi* ($hrpN_{Ech}$) (7), and *R. solanacearum* (*popA*) (4) are located adjacent to or near their respective *hrp* clusters, whereas the *P. syringae hrpZ* gene resides within a *hrp* operon (31). *E. chrysanthemi hrpN* mutants are reduced in infectivity at low inoculum levels and are unable to elicit the HR (7), but harpin gene mutations in *E. amylovora* CFBP1430 (a highly virulent strain) (5), *R. solanacearum* (4), and *P. syringae* (1) produce weak phenotypes or no phenotype. Thus, individual harpins do not appear to be necessary for elicitation of the HR by most bacteria. The potential role of harpins in determining host specificity is uncertain. PopA may be a host specificity factor because the isolated protein elicits the HR selectively in those plants in which *R. solanacearum* also elicits the HR, whereas the isolated harpins from *E. amylovora* and three *P. syringae* pathovars trigger the HR in various plants in a manner that shows no relationship to bacterial host range (30, 31, 60, 79). Harpin activity may involve interactions with plant cell walls. The HrpZ harpin binds to the walls of intact plant cells but not to protoplasts, and it also fails to trigger HR-associated responses in protoplasts (33). The elicitor activity of harpins is unlikely to be enzymatically based because various fragments retain activity (1, 4, 45).

The function of the *P. syringae* HrpZ harpin is particularly puzzling. Several observations suggest a simple, direct role for HrpZ in HR elicitation. HrpZ is the predominant protein secreted by the *P. syringae* Hrp system in culture (31, 88), the *hrpZ* gene is conserved in divergent *P. syringae* pathovars (60), and the isolated protein elicits an apparent programmed cell death in plants that is indistinguishable from the HR elicited by living bacteria (31). Furthermore, *hrpZ* deletion mutations in the cosmid pHIR11 functional cluster of *P. syringae* pv. syringae *hrp* genes strongly reduce the HR elicitation activity of *E. coli* cells carrying only pHIR11. The same mutation only slightly reduces the HR in *P. syringae* pv. syringae, but this can be explained by postulating the existence of a second harpin encoded elsewhere in the bacterial genome (1).

However, other observations show that the relationship of HrpZ to HR elicitation is more complex. Mutation of *hrmA* (32, 35), which is in a variable region flanking the conserved *hrp* cluster in pHIR11, abolishes HR activity in tobacco without diminishing HrpZ synthesis or secretion (1). Thus, isolated HrpZ is sufficient to elicit an HR in tobacco leaves but HrpZ produced by bacteria in plants is not. Instead, HrmA, with no apparent function in the Hrp secretion apparatus, is necessary for bacterial elicitation of the HR, and thus, HrmA appears to be the actual elicitor of the HR produced by bacteria carrying pHIR11. HrmA has several characteristics of an Avr protein (3). Avr proteins and the role of the Hrp system (and possibly harpins) in their delivery into plant cells will be discussed below.

HrpA pilin and other secreted proteins. *P. syringae* pv. tomato DC3000 secretes at least four proteins in addition to HrpZ into the medium in a Hrp-dependent manner (88). One of these is the 10-kDa product of *hrpA*, which forms a 6- to 8-nm-diameter "Hrp pilus" (61). A nonpolar *hrpA* mutant no longer elicits the HR in appropriate test plants, even when carrying an *avr* gene known to interact with an *R* gene in the plant. It thus appears that the Hrp pilus is essential for the delivery of Avr signals (discussed further below). Although it is not known whether the Hrp pilus functions primarily in bacterial attachment or as a conduit for the delivery of bacterial proteins across the plant cell wall, it is interesting that *Agrobacterium tumefaciens* requires a pilus similar in size (3.8-nm diameter) to transfer T-DNA and the VirE2 protein into plant cells (24).

Hrp DELIVERY OF AVR PROTEINS INTO PLANT CELLS

***avr* genes and their products.** In fundamental contrast to the *hrp* genes, *avr* genes are scattered in their distribution among strains of plant pathogenic bacteria (20, 46). More than 30 bacterial *avr* genes have been cloned from *P. syringae* and *X. campestris*, but until recently, characterization of the menagerie of encoded proteins has largely defined what these proteins do not do. Isolated Avr proteins do not elicit any responses when they infiltrate plant leaves. They do not appear to be secreted in culture and are hydrophilic proteins lacking N-terminal signal peptides or other recognizable secretion signals (properties consistent with potential secretion by the type III pathway). They do not have demonstrable enzymatic activity (with the exception of AvrD, which directs the synthesis of syringolide elicitors of an *R* gene-dependent HR [55]), and the majority of them do not contribute in an obvious way to parasitic fitness or virulence in the infection of cultivars lacking a matching *R* gene that would trigger the HR. However, there are several significant exceptions to the last point (20, 46) and there is growing evidence that Avr proteins have a primary function in virulence, even though the HR-triggering effects of *Avr-R* interactions are epistatic over these virulence functions. How Avr proteins might promote parasitism is mysterious, but support for such a primary role comes from observations that their action is dependent on the Hrp system and their site of action is within host cells. The next two sections address these points and provide evidence that the main function of the Hrp system is in the delivery of Avr-like proteins into plant cells.

Hrp dependency of Avr phenotypes. *avr* genes have no phenotype when expressed in *hrp* mutant pathogens or in nonpathogenic bacteria like *E. coli*, which lack the Hrp system (highly expressed *avrD* is the sole exception to the latter point [40]). For many *avr* genes, especially those in *P. syringae*, one simple explanation is that their expression is dependent on Hrp regulatory factors (46). However, expression of *avr* genes from vector promoters does not obviate the requirement for a functional Hrp system. The recent finding that the functional cluster of *P. syringae* pv. syringae *hrp* genes carried on cosmid pHIR11 is sufficient to deliver heterologous *avr* gene signals indicates the fundamental interdependency of Hrp and Avr functions in bacterial elicitation of the HR (28, 58). A key property of pHIR11 enabling this discovery is that the cosmid confers on nonpathogenic *E. coli* and *P. fluorescens* the ability to elicit the HR in tobacco and several other plants, but it is ineffective in doing so in soybean and *Arabidopsis*. The simplest explanation is that *hrmA*, which is carried on pHIR11 and has several properties of *avr* genes (3), interacts with an unknown *R* gene in tobacco but with no *R* genes in soybean and *Arabidopsis*. This suggested that expression of appropriate *avr* genes in *trans* would enable nonpathogens carrying pHIR11 to elicit an *R* gene-dependent HR in soybean, *Arabidopsis*, and other plants. Indeed, this was observed with *avrB* (from *P. syringae* pv. glycinea) and five other *P. syringae avr* genes (28, 58).

The ability of pHIR11 to deliver *avr* gene signals requires HrcC (absolutely) and HrpZ (variably) (28, 58). The inability of HrpZ to support AvrB signal delivery when supplied exogenously indicates that the harpin has a role only when produced along with AvrB and therefore may be an extracellular accessory in the delivery of Avr proteins, as YopD is in the

delivery of YopE (28, 62). Most importantly, these experiments reveal that a functional Hrp secretion system is required for the delivery of several *avr* gene signals. Furthermore, the use of promoters different in strength and of epitope-tagged AvrB revealed that the requirement for a functional Hrp secretion system cannot be obviated by high levels of AvrB in the bacterial cytoplasm or by infiltration of leaves with purified AvrB at a level 1,000-fold higher than that required by living Hrp^+ bacteria to elicit the HR (28). Thus, AvrB does not appear to act in the bacterial cytoplasm or in leaf intercellular spaces. These observations strongly support the hypothesis, depicted in Fig. 2, that the type III protein secretion system in plant pathogens, as in animal pathogens, is capable of delivering bacterial proteins into host cells.

Demonstrations of Avr action in host cells. Bacterial transfer of Avr proteins into plant cells has not been observed directly. However, there is evidence that several of these proteins are biologically active when produced within plant cells, that the HR-triggering activity of one of them is dependent on physical interaction with its cognate plant *R* gene product, and that the activity of another is dependent on localization to the plant cell nucleus. AvrB action in plant cells was demonstrated with *Arabidopsis* plants carrying the cognate *RPM1 R* gene (28). An *Arabidopsis rpm1* mutant was transformed with *avrB* and crossed with a wild-type line, thus producing seedling progeny carrying both *avrB* and *RPM1* which died soon after germinating. One symptomless *rpm1* mutant transgenic plant was obtained; this individual expressed relatively low levels of an *avrB* construct carrying the PR-1a plant protein signal peptide, with the likely consequence that the plant cytoplasm would be exposed only transiently or to low levels of AvrB. The properties of this survivor suggest that plants are sensitive to AvrB even in the absence of a functional matching *R* gene and that vanishingly low levels of the protein are sufficient to elicit the HR in the presence of a complete *R* gene. A biolistic, transient expression assay revealed that *avrB* lacking a signal peptide (and therefore localized to the plant cytoplasm) was lethal to *Arabidopsis* leaf cells carrying *RPM1* but not to those lacking the *R* gene (28). This approach was extended with *avrRpt2* (from *P. syringae* pv. tomato) (47). Similarly, an *A. tumefaciens* transient expression system was used to deliver *avrPto* (from *P. syringae* pv. tomato) and *avrBs3* (from *X. campestris* pv. vesicatoria) into plants, resulting in an *R* gene-dependent HR in all cases (66, 70, 72). Thus, whereas no bacterial Avr protein has been observed to have an effect when delivered exclusively to the surface of plant cells, all four of those tested elicit an *R* gene-dependent response when expressed inside them.

The simplest model for the molecular basis of gene-for-gene HR elicitation predicts physical interaction between the protein products of cognate *avr* and *R* genes. This has been observed with the bacterial AvrPto and plant Pto proteins; mutations in the molecular partners that diminish physical interaction in the yeast two-hybrid system also diminish biological function (66, 70). Because AvrPto action requires a functional Hrp system in either *P. syringae* pv. tomato (64) or nonpathogens carrying the pHIR11 *hrp* cluster (28, 58) and it involves physical interaction with a cytoplasmic target in the host, the Hrp-mediated transfer of AvrPto into plant cells seems certain.

While many bacterial Avr proteins appear to be targeted to the host plant cytoplasm, members of the AvrBs3 family in *Xanthomonas* spp. are targeted to the host nucleus. These proteins carry functional nuclear localization signals (NLS) in the C-terminal region (72, 86). When fusions of this C-terminal region and a *uidA* reporter are transiently expressed in onion epidermal cells by biolistic bombardment, β-glucuronidase activity is localized to the nucleus (72, 86). Deletion of all three of the NLS sequences abolishes nuclear localization in the biolistics assay and HR elicitation by *X. campestris* pv. vesicatoria cells in pepper plants carrying the *Bs3 R* gene, and both of these abilities can be restored by substitution of the simian virus 40 large-T antigen NLS (72). These results suggest that the *Bs3* product must also be localized to the nucleus, but because this *R* gene has not been cloned, this awaits confirmation.

Gaps in our knowledge of the Hrp pathway and the inventory of its protein traffic. Although the rings of evidence that the Hrp system transfers Avr proteins into plant cells are collectively strong, there are formal gaps in each. (i) In the system explored in the most detail, AvrPto-Pto, physical interaction between the bacterial and plant proteins has not been demonstrated in vivo, and a second host protein, Prf, is required for AvrPto-Pto-mediated HR elicitation. Furthermore, all of the other cloned plant *R* genes that interact with known bacterial *avr* genes resemble Prf (a nucleotide-binding site leucine-rich repeat protein) rather than Pto (a kinase) (68). (ii) R proteins appear to be present at vanishingly low levels, and none has been directly observed in the cytoplasm, although RPS2 localizes to the cytoplasm-equivalent fraction in a rabbit reticulocyte dog pancreatic microsome in vitro translation-translocation system (47). (iii) Similarly, Avr proteins appear to be effective at vanishingly low levels (28) and immunogold labeling and electron microscopy of infected plant tissues has revealed their presence only in bacterial cells (13, 87). (iv) Finally, no Avr protein has been directly shown to be translocated out of the bacterial cytoplasm in culture by the Hrp system. It is worth noting that the *A. tumefaciens* VirE2 protein has never been observed to be transferred into plant cells, although the indirect evidence for its action within plant cells seems irrefutable (89).

Many (if not most) of the genes encoding proteins that are transferred into plant cells by these bacterial pathogens probably await discovery. Systematic completion of the inventory is thwarted by two problems. First, the contribution of the genes to virulent interactions may be too subtle for detection in mutant screens, and cognate *R* genes that would reveal Avr phenotypes when the bacterial genes are heterologously expressed may be unknown or nonexistent. Second, no plant signals or regulatory mutants have been found that permit bacteria to secrete these proteins in culture, although harpins, pilins, and possibly other proteins that serve the type III secretion system are secreted in culture. A critical feature of the type III protein secretion system in *Yersinia* spp. is its capacity to withhold full secretion of virulence proteins until contact with the host cell (18). The fact that nonpathogens carrying the pHIR11 functional *hrp* cluster secrete HrpZ but not AvrB in culture (28) indicates that the genetic information for this expected regulatory step is carried within the *hrp* cluster and is therefore subject to discovery through systematic analysis of the *hrp* genes. Obtaining Avr protein secretion in culture is important because (i) it is likely to be associated with structures that normally are used to penetrate the plant cell wall (and possibly trigger host cell endocytosis) and therefore will yield clues to the transfer process and (ii) it will allow proteins targeted to the host to be systematically characterized through identification of novel proteins in the medium. The exploration of DNA sequences flanking *hrp* clusters also should be useful in this search because of the growing evidence that these regions are enriched in genes whose products probably travel the Hrp pathway (51, 53, 54).

A new designation for effector proteins that are delivered by the Hrp system to plant cells would be useful: Avr appears

to be inappropriate because some of the encoding genes may have no Avr phenotype and the primary function of Avr proteins is almost certainly in virulence, not avirulence. One proposal is to designate new members of this class Hop (Hrp-dependent outer protein) and to add a four-letter suffix identifying the bacterial species, pathovar, and gene, based on the current system for uniform nomenclature of avirulence genes (3, 75). For example, the gene encoding a newly found *P. syringae* pv. syringae protein in this class would be designated *hopPsyA*. Hop is analogous to the Yop (Yersinia outer protein) designation for proteins secreted by the prototypical *Yersinia* type III secretion system but is broadened here for consistency with the use of Hrp and Avr for plant pathogens in all genera.

A NEW PARADIGM AND FUTURE EXPLORATIONS

Pathogenesis based on the Hrp delivery of Avr-like (Hop) proteins into host cells (depicted in Fig. 2) provides a simple and unifying explanation for many characteristics of plant pathogenic *Erwinia*, *Pseudomonas*, *Xanthomonas*, and *Ralstonia* spp. (2). These include the one-to-one relationship between bacterial cells and HR-responding plant cells (expected with contact-dependent secretion), the gene-for-gene interactions of pathogen races and host cultivars (expected if *avr* and *R* gene products can directly interact within host cells), and the enormous diversity in host range and other pathogenic attributes among closely related strains (expected with a pool of horizontally transferable and interchangeable genes whose products can either promote or betray parasites in coevolving hosts). The latter point is particularly relevant to *P. syringae* and *X. campestris*, which are divided into more than 40 and 140 pathovars, respectively. And it is consistent with the location of many *avr* genes on plasmids and the ability of *avr* genes to function with heterologous Hrp systems (20). In this regard, one potential difference between the type III systems of animal and plant pathogens is noteworthy. In animal pathogen type III systems, the secretion of many effector proteins requires customized chaperones, which are often encoded by genes linked to effector genes (76). The ability of many isolated *avr* genes to function heterologously in other pathogens or in nonpathogens carrying the pHIR11 functional *hrp* cluster suggests that Avr protein delivery does not require specific chaperones or that a promiscuous chaperone gene exists within the *hrp* cluster.

This new model of plant pathogenicity invites several fundamental questions in plant pathology and pathogenic microbiology in addition to those discussed above regarding the Hrp system and the identification of its traffic. How do Hrp-delivered proteins alter host metabolism to promote bacterial growth in plant intercellular spaces? How is host specificity determined at the pathovar-host species level? That is, are *avr-R* gene interactions important here also, as suggested by the discovery of novel *avr* genes through expression in heterologous pathovars (44, 83), or do Avr-like proteins have important positive effects in bacterial adaptation to host species? Given the use of homologous secretion systems, how similar are the functions of the virulence proteins that plant and animal pathogens transfer into their hosts? Sequence similarities involving secreted *Yersinia* proteins have been noted only between YopN and YopJ and the *E. amylovora* HrpJ and *X. campestris* pv. vesicatoria AvrRxv proteins, respectively (10, 46). Since YopN appears to be an extracellular component of the secretion system and the effector activity of YopJ is unknown, this key question remains unanswered. Further comparisons should give us a broader perspective on the evolution of bacterial pathogenicity and may lead to unanticipated controls for diseases of both plants and animals.

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Isolation of the *hreX* Gene Encoding the HR Elicitor Harpin (Xcp) from *Xanthamonas campestris* pv. *pelargonii.* S. SWANSON and Z-M. Wei. EDEN Bioscience Corporation, Bothell, WA 98011 USA. Phytopathology 90:S75. Publication no. P-2000-0537-AMA.

This study reports the isolation of a gene encoding a proteinaceous HR elicitor from *Xanthomonas campestris* pv. *pelargonii, Xcp*. The HR elicitor exhibits a high potency for eliciting HR in tobacco. Treatment of the *Xcp* HR Elicitor with proteases resulted in a loss of HR activity. Degenerate oligonucleotides were designed based on amino acid sequences obtained from the purified HR elicitor and used to screen a *Xanthomonas campestris* pv. *pelargonii* genomic library. An open reading frame, ORF, was identified consisting of 381 base pairs that encoded a protein of 126 amino acids. The ORF initiated with a typical methionine start codon and was preceded by a putative ribosome-binding site. The ORF was designated as the *hreX* gene, encoding the HR elicitor harpin (Xcp). HreX has a molecular weight of 13.3KD, a theoretical pI of 3.8 and is glycine rich. Further studies of harpin (Xcp) and its bioactivity are currently underway.

Isolation of the *hreX* Gene Encoding the HR Elicitor Harpin$_{Xcp}$ from *Xanthamonas campestris* pv. *pelargonii*.

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INTRODUCTION

The last decade has been witness to the identification, isolation and characterization of a number of hypersensitive response elicitors from plant pathogens (HR elicitors). HR elicitors have been reported from species of *Erwinia*, *Pseudomonas*, and *Ralstonia*. For the most part, the HR elicitors reported have been classified as belonging to the harpin protein family. Harpin proteins elicit the hypersensitive response in tobacco and other non-host plants, and are glycine rich, and heat-stable. They are secreted via a type III secretion system. Harpin proteins are generally located in or approximate to an *hrp* gene cluster, a highly conserved region of genes that encode regulatory factors and other components of the type III secretion system. In most of the species above, identification of the *hrp* clusters preceded the identification of the harpin proteins. The *hrp* gene clusters from several species of *Xanthomonas* have also been identified and highly studied, though to date an HR elicitor from *Xanthomonas* has not been reported. The current study reports the isolation of a gene encoding an HR elicitor from *Xanthomonas campestris pv. pelargonii* (*Xcp*).

PROTEINACEOUS HR ELICITOR

Figure 1.
Wild type *Xcp* was grown in a minimal media that was optimized to induce the expression of the HR elicitor. The induced culture was centrifuged, and the cell pellet resuspended and sonicated. The cellulose was then heat-treated, cooled and centrifuged. The resulting supernatant, or cell-free elicitor preparation (CFEP), was infiltrated into tobacco leaves and a hypersensitive response was observed. When samples of the CFEP were incubated in the presence of a protease prior to infiltration, no symptoms of HR were observed in the tobacco plant.

IDENTIFICATION AND ISOLATION OF HR ELICITOR

Figure 2.
CFEP was used as the starting material for the chromatographic purification of the HR elicitor. Purification consisted of two hydrophobic interaction steps: a cation exchange step, and a reverse phase chromatography polishing step (RPC). The final RPC step yielded a nearly homogeneous *Xcp* HR elicitor.
Lane A, CFEP loaded on to Butyl Soph.; lane B, HR active fraction from the Butyl Soph. loaded into Mono S; lane C, HR active fraction from Mono S loaded on to Phenyl Soph.; lane D, HR active fraction from Phenyl Soph. loaded on to an RPC column; lane E, HR active fraction from the final RPC step, representing the purified *Xcp* HR elicitor.

GENE ISOLATION

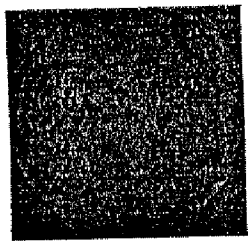


Figure 3.
The purified HR elicitor was proteolytically digested and the resulting peptides separated. Amino acid sequencing of one of the peptides yielded an unambiguous sequence of nine amino acids. A fully degenerate pool of oligonucleotides was made based on the amino acid sequence. The oligonucleotide pool was 5' end radiolabeled and used as a probe against an *Xcp* genomic library.
The *Xcp* genomic library was constructed in pBluescript II that contained 9 to 12 kb *Xcp* Sau3 A1 genomic DNA fragments. The library was plated and the DNA transferred to nylon membranes and fixed. The membranes were hybridized with the previously described probe. Approximately 7000 colonies were screened, and 30 colonies were isolated for further analysis. Examples of positive hybridizing DNA from library constructs are shown.

Figure 4.
Plasmids were isolated from colonies that showed positive hybridization with the HR elicitor probe. A Southern analysis was performed on the digested plasmids in order to confirm the results of the colony hybridization (Figure 3). Samples 2, 4, and 6 were further analyzed.

Figure 5.
Genomic DNA inserts from samples 2, 4, and 6 (Figure 4) were digested and subcloned. The subclones were constructed in pBluescript II and contained 1.5 to 3 kb genomic inserts. A final Southern analysis was performed on the subclones. This confirmed the presence of the genomic DNA fragments which hybridized with the HR elicitor probe.

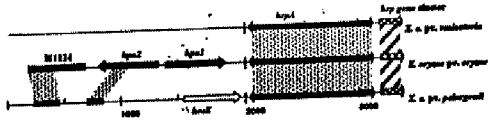


Figure 6.
DNA sequencing was performed on the inserts of the final constructs which hybridized with the HR elicitor probe (Figure 5; samples 2a, 4a, and 6a). A 378 base pair open-reading-frame (ORF) encoded a 126 amino acid protein was identified. The deduced protein had a molecular weight of ca. 12 kDa, a theoretical pI of 3.5, and was glycine rich. These characteristics match those of the originally purified *Xcp* HR elicitor protein. The ORF was designated *hreX*. The *hreX* gene product, HreX, consisted of 15.9% glycine, 10.4% glutamine, 11.4% serine, and one cysteine residue (0.9%). Sequencing of the regions flanking of *hreX* revealed that *hreX* was located immediately downstream of a *hrpA* homologue. Based on *hrp* gene clusters reported for other *Xanthomonas* species, it would seem likely that *hreX* is located just outside of the *hrp* gene cluster. BLAST analysis did not identify any genes with significant sequence similarities to the *hreX* gene. Significant homologies were observed between regions upstream of the *hreX* gene and I81114 and *hpa2* from *X. oryzae pv. oryzae* (1).

hreX HOMOLOGUE

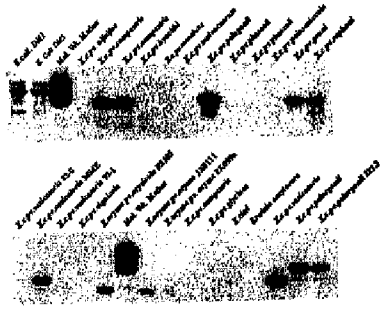
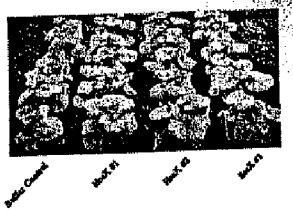


Figure 7.
Various species of *Xanthomonas* and other bacteria were obtained and screened for the presence of an *hreX* homologue. Southern analyses were performed with *hreX* probe. Hybridization with the *hreX* probe was observed in the genomic DNA of the bacterial species appearing in bold type.

BIOACTIVITY OF HREX

HreX was expressed in *E. coli*, and proposed to be identical isoform to the previously described CFEP, to generate a crude HreX extract. When infiltrated into tobacco and several other plant species, the HreX extract induced the hypersensitive response. Experiments have also been conducted with crude HreX extract to evaluate its ability to induce resistance to tobacco mosaic virus and to induce plant growth enhancement in tomato. Three days after the treatment of tobacco plants with the crude HreX extract, tobacco plants were inoculated with tobacco mosaic virus (TMV). Four days after TMV inoculation, the treated plants were observed to have 75% fewer TMV-caused lesions compared to the buffer treated control plants. Tomato plants grown from seed soaked in the crude HreX extract were observed to have an average growth 15-20% greater than the buffer treated control plants (determined by measuring plant height).

Figure 8.
Additional experiments were conducted with HreX to evaluate its ability to induce disease resistance in geraniums. Geraniums (variety Rose Star) were treated with varying concentrations of a crude HreX extract (HreX #1, 2, & 3). Five days after treatment, the geranium plants were inoculated with *Xanthomonas campestris pv. pelargonii* (100 ml at $1x10^6$ cfu per ml). Fifteen days after inoculation, the HreX solution was determined to be effective in controlling the disease.



CONCLUSIONS

- A hypersensitive response elicitor was purified to near homogeneity from *Xanthomonas campestris pv. pelargonii*.
- The gene corresponding to the *Xcp* HR elicitor was isolated and designated *hreX*.
- The *hreX* gene is located next to *hrpA* just outside of the hrp gene cluster.
- BLAST analysis did not identify any genes with significant sequence similarities to the *hreX* gene. Significant homologies were observed between regions upstream of the *hreX* gene and I81114 and *hpa2* from *X. oryzae pv. oryzae* (1).
- Sequences similar to *hreX* have been identified by Southern analysis in various other species of *Xanthomonas* including some pathovars of *X. c. pv. vesicatoria* and *X. oryzae*.
- HreX was expressed in *E.coli* and induced a hypersensitive response when infiltrated into tobacco.
- Treatment of tobacco and geranium plants with a crude HreX extract resulted in increased disease resistance.
- Treatment of tomato plant with the HreX extract resulted in plant growth enhancement.
- Studies are currently underway to determine whether HreX is secreted via the type III secretion system.

1. Zhu, W., Magbanua, M. M., and F. F. White. 2000. Identification of Two Novel Hrp-Associated Genes in *hrp* Gene Cluster of *Xanthomonas oryzae* pv. *oryzae*. J. Bacteriol. 179:5655-5662.

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Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria

Sir,

Genes of plant-pathogenic bacteria controlling hypersensitive response (HR) elicitation and pathogenesis were designated '*hrp*' by Lindgren *et al.* in 1986 (*J Bacteriol* **168:** 512–522). *hrp* genes have been characterized in several species of the four major genera of Gram-negative plant pathogens, *Erwinia*, *Pseudomonas*, *Ralstonia* (a new proposed genus including *Pseudomonas solanacearum*) and *Xanthomonas*. To date, *hrp* genes have been found mainly in large clusters, and they have been shown to be conserved physically and, in many cases, functionally among different bacteria. Hybridization studies and genetic analyses have revealed the presence of functional *hrp* genes even in species that are not typically observed to elicit an HR, such as *Erwinia chrysanthemi* and *Erwinia stewartii*, suggesting that *hrp* genes may be common to all Gram-negative plant pathogens, possibly excluding *Agrobacterium* spp. Current knowledge of *hrp* genes has been reviewed by Bonas (1994, *Curr Top Microbiol Immunol* **192:** 79–98) and by Van Gijsegem *et al.* (1995, In *Pathogenesis and Host–Parasite Specificity in Plant Diseases: Histopathological, Biochemical, Genetic and Molecular Basis.* Volume 1. (Kohmoto *et al.*, eds); Oxford: Pergamon Press, pp. 273–292).

The nucleotide sequences of four *hrp* gene clusters, those of *Ralstonia solanacearum* (previously *P. solanacearum*) (Genin *et al.*, 1992, *Mol Microbiol* **6:** 3065–3076; Gough *et al.*, 1992, *Mol Plant–Microbe Interact* **5:** 384–389; Gough *et al.*, 1993, *Mol Gen Genet* **239:** 378–392; Van Gijsegem *et al.*, 1995, *Mol Microbiol* **15:** 1095–1114), *Erwinia amylovora* (Bogdanove *et al.*, 1996, *J Bacteriol* **178:** 1720–1730; Wei and Beer, 1993, *J Bacteriol* **175:** 7958–7967; Wei and Beer, 1995, *J Bacteriol* **177:** 6201–6210; Wei *et al.*, 1992, *Science* **257:** 85–88; S. V. Beer, unpublished), *Pseudomonas syringae* pv. *syringae* (Huang *et al.*, 1992, *J Bacteriol* **174:** 6878–6885; Huang *et al.*, 1993, *Mol Plant–Microbe Interact* **6:** 515–520; Huang *et al.*, 1995, *Mol Plant–Microbe Interact* **8:** 733–746; Lidell and Hutcheson, 1994, *Mol Plant–Microbe Interact* **7:** 488–497; Preston *et al.*, 1995, *Mol Plant–Microbe Interact* **8:** 717–732; Xiao *et al.*, 1994, *J Bacteriol* **176:** 1025–1036), and *Xanthomonas campestris* pv. *vesicatoria* (Fenselau *et al.*, 1992, *Mol Plant–Microbe Interact* **5:** 390–396; Fenselau and Bonas, 1995, *Mol Plant–Microbe Interact* **8:** 845–854; U. Bonas, unpublished), have been largely determined. These clusters each contain more than twenty genes, many of which encode components of a novel protein-secretion pathway designated 'type III'. It has been shown directly that various extracellular proteins involved in pathogenesis and defence elicitation by plant-pathogenic bacteria utilize this pathway (Arlat *et al.*, 1994, *EMBO J* **13:** 543–553; He *et al.*, 1993, *Cell* **73:** 1255–1266; Wei and Beer, 1993, ibid.), and the pathway is known to function in the export of virulence factors from the animal pathogens *Salmonella typhimurium*, *Shigella flexneri*, and *Yersinia enterocolitica*, *Yersinia pestis*, and *Yersinia pseudotuberculosis* (for reviews, see Salmond and Reeves, 1993, *Trends Biochem Sci* **18:** 7–12; and Van Gijsegem *et al.*, 1993, *Trends Microbiol* **1:** 175–180). Nine type III secretion genes are conserved among all four of the plant pathogens listed above and among the animal pathogens. Based on sequence analysis and some experimental evidence, they are believed to encode one outer-membrane protein, one outer-membrane-associated lipoprotein, five inner-membrane proteins, and two cytoplasmic proteins, one of which is a putative ATPase. All of the predicted gene products, except the outer-membrane protein, show significant similarity to components of the flagellar biogenesis complex (for reviews see Blair, 1995, *Annu Rev Microbiol* **49:** 489–522; and Bischoff and Ordal, 1992, *Mol Microbiol* **6:** 23–28). We herein refer to the *hrp*-encoded type III pathway as the 'Hrp pathway'.

Because *hrp* genes have been characterized independently in diverse plant-pathogenic bacteria, *hrp* gene nomenclature differs in different species, and it is not always consistent even within the same organism. Different designations are used for homologous genes, and, even worse, the same designation is used for different genes in different organisms. For example, *hrpI* of *E. amylovora* is homologous with *hrpC2* of *X. campestris* pv. *vesicatoria* and *hrpO* of *R. solanacearum*, and the homologue in *P. syringae* pv. *syringae* appears in the literature both as *hrpI* and as *hrpJ2*. Also, '*hrpN*' in *R. solanacearum* designates a secretion-pathway gene, whereas in *E. amylovora*, '*hrpN*' designates the gene encoding the elicitor harpin. Furthermore, in many bacteria the number of known *hrp* genes approaches 26. In anticipation of exhausting the alphabet, some authors chose to designate *hrp* genes with a letter and a number, creating the potential for confusion of distinct genes with alleles of the same gene. For *hrp* gene researchers, the current nomenclature is at best inconvenient; for other scientists, it is bewildering.

Another problem exists: accumulation of knowledge about the structure of *hrp* loci has outpaced the accumu-

lation of information regarding the specific functions of individual genes. Typically, *hrp* loci have been identified by polar, transposon mutagenesis. Conceivably, a particular gene within an operon required for the Hrp phenotype may not be a strict Hrp determinant, but may play a more subtle role. Moreover, even phenotypes of mutations in well-characterized *hrp* genes are not the same in all pathogens. For example, although the *hrpN* gene of *E. amylovora* is required for pathogenesis of pear fruit, the homologous gene in *E. stewartii* (D. L. Coplin, unpublished) is dispensible for pathogenicity of corn. In the macerogenic bacterium *E. chrysanthemi*, even polar mutations that disrupt *hrp* secretion altogether only reduce the apparent frequency of successful infection initiation (Bauer *et al.*, 1994, *Mol Plant-Microbe Interact* **7**: 573–581). Thus, the designation '*hrp*' in its strict sense, i.e., meaning required for the HR and pathogenicity, is not uniformly applicable.

At the 7th International Congress on Molecular Plant–Microbe Interactions held in Edinburgh, Scotland in 1994, a committee of *hrp* researchers and others was formed to address these problems. We, the committee members, agreed upon a system to standardize names for the subset of *hrp* genes that are broadly conserved, and agreed to broaden the definition of the '*hrp*' designation, as follows.

For the subset of *hrp* genes that are broadly conserved, the new, unique, lower-case symbol '*hrc*' will be used. The '*hr*' of *hrp* has been retained in order to evoke that name, and the '*c*' has been added to denote 'conserved.' The upper-case designations will correspond to those of the type III secretion genes of *Yersinia* spp. (for a review, see Forsberg *et al.*, 1994, *Trends in Microbiol* **2**: 14–19), *yscC, yscJ, yscN, yscQ–U*, and *lcrD*, except that the *lcrD* homologues will be designated '*hrcV*' to avoid confusion of these as homologues of *yscD*, which is another, less well-conserved type III gene of *Yersinia* spp. We request that *Yersinia* researchers omit the letter '*V*' in naming any new *ysc* genes that might be discovered. The *ysc* nomenclature was chosen as a standard for revising *hrp* gene names for its convenient uniformity, and because, of all the genes that comprize the several known type III systems, the *Yersinia* genes show the highest degree of sequence similarity to the type III (*hrp*) genes of plant pathogens. The new names for the nine genes are given in Table 1, along with the current names in *R. solanacearum*, *E. amylovora*, *P. syringae* pv. *syringae*, and *X. campestris* pv. *vesicatoria*, and the names of homologues involved in flagellar biogenesis.

In designating genes as '*hrc*', 'broadly conserved' genes were defined as being present among the *hrp* genes of at least one representative species of each of the four plant-pathogenic genera discussed here and among the type III genes of each of the animal-pathogenic species *S. typhimurium*, *S. flexneri*, and the three yersiniae. Gene families were defined based on pairwise sequence alignments. Any two genes were considered homologous if a BESTFIT alignment (Devereux *et al.*, 1984, *Nucl Acids Res* **12**: 387–395) of the predicted amino acid sequences using default parameters yielded a quality score at least five times the standard deviation above the mean quality score of 100 alignments, for each of which one of the sequences had been randomized prior to alignment (Doolittle, 1986, *Of URFs and ORFs: a Primer on How to Analyse Derived Amino Acid Sequences*. Mill Valley, California: University Science Books).

Genes that did not meet the criterion for the '*hrc*' designation will remain '*hrp*'. We have chosen to use this criterion until more data regarding structure and precise function of the products of the *hrp* and other type III genes becomes available. Some of the genes that did not meet the criterion in fact may be common to *Ralstonia*, *Erwinia*, *Pseudomonas, and Xanthomonas*, and have homologues in the animal pathogens, yet may be sufficiently diverged to obscure obvious homology by direct sequence comparison. As structural and functional data accrue, such relationships may become clear, and the list of *hrc* genes

Table 1. Current names and new, unified names for the broadly conserved *hrp* genes of *R. solanacearum*, *E. amylovora*, *P. syringae* pv. *syringae*, and *X. campestris* pv. *vesicatoria*. Homologues that function in flagellar biogenesis are given in the bottom row.

Unified	*hrcC*	*hrcJ*	*hrcN*	*hrcQ*	*hrcR*	*hrcS*	*hrcT*	*hrcU*	*hrcV*
R. solanacearum[a]	*hrpA*	*hrpI*	*hrpE*	*hrpQ*	*hrpT*	*hrpU*	*hrpC*	*hrpN*	*hrpO*
E. amylovora[b]	*hrcC*	*hrcJ*	*hrcN*	*hrcQ*	*hrcR*	*hrcS*	*hrcT*	*hrcU*	*(hrpI) hrcV*
P. syringae[c]	*hrpH*	*hrpC*	*hrpJ4*	*hrpU2/U*	*hrpW*	*hrpO*	*hrpX*	*hrpY*	*(hrpJ2) hrpI*
X. campestris[d]	*hrpA1*	*hrpB3*	*hrpB6*	*hrpD1*	*hrpD2*	*hrpD3*	*hrpB8*	*hrpC1*	*hrpC2*
(Flagellar)[e]		*flif*	*flil*	*fliY,N*	*fliP*	*fliQ*	*fliR*	*flhB*	*flhA*

a. Gough *et al.*, 1992, ibid.; Gough *et al.*, 1993, ibid.; Van Gijsegem *et al.*, 1995, ibid.

b. Bogdanove *et al.*, 1996, ibid.; Wei and Beer, 1993, ibid.; S. V. Beer, unpublished.

c. Huang *et al.*, 1992, ibid.; Huang *et al.*, *Mol Plant-Microbe Interact* **6**: 515–520, 1993; Huang *et al.*, 1995, ibid.; Lidell and Hutcheson, *Mol Plant-Microbe Interact* **7**: 488–497, 1994; Preston *et al.*, 1995, ibid. The predicted product of *hrpU2* aligns with the N-terminal two-thirds of a multiple alignment of the other plant- and animal-pathogen homologues; that of *hrpU* aligns with the remaining N-terminal one-third. Respectively, these genes will be designated '$hrcQ_A$' and '$hrcQ_B$.'

d. Fenselau *et al.*, 1992, ibid.; Fenselau and Bonas, 1995, ibid.; U. Bonas, unpublished. Hwang *et al.* (1992, *J Bacteriol* **174**: 1923–1931) published the sequence of two genes from *Xanthomonas campestris* pv. *glycines*, designated 'ORF1' and 'ORF2,' that are homologous to hrpD1 and hrpD2 of *X. campestris* pv. *vesicatoria*, respectively.

e. For reviews, see Blair (1995, ibid.) and Bischoff and Ordal (1992, ibid.).

may grow. For any new *hrp* genes that may be discovered, we recommend the strict, sequence-alignment-based criterion for use of the '*hrc*' designation until sufficient structural and functional studies can be completed.

Some *hrp* genes are conserved only within subgroups of plant pathogens. One example is the regulatory gene *hrpB* of *R. solanacearum* (Genin *et al.*, 1992, ibid.). This gene, a member of the *araC* family, is present also in pathovars of *X. campestris* (Kamdar *et al.*, 1993, *J Bacteriol* **175:** 2017–2025; Kamoun and Kado, 1990, *J Bacteriol* **172:** 5165–5172; U. Bonas, unpublished), but absent from the *hrp* gene clusters of *P. syringae* and *E. amylovora*, which contain regulatory genes that are members of the two-component regulatory-system family (Grimm *et al.*, 1995, *Mol Microbiol* **15:** 155–165; Grimm and Panopoulos, 1989, *J Bacteriol* **171:** 5031–5038; Xiao *et al.*, 1994, ibid.; S. V. Beer, unpublished). As another example, the *hrp* gene clusters of *P. syringae* and *E. amylovora* each contain a homologue of the *Yersinia* gene *yopN* (Bogdanove *et al.*, 1996, ibid.), yet no homologue of this gene has been found in *R. solanacearum* or *X. campestris.* It is noteworthy that the genetic organizations of the *hrp* gene clusters of *X. campestris* and *R. solanacearum* are quite similar to, yet distinct from, those of *P. syringae* and *E. amylovora*, which resemble one another. We will not attempt a nomenclatural revision here for any of the non-*hrc* genes, but we encourage authors, wherever possible, to standardize names for such genes, at least within these subgroups, by using conventional rules for bacterial genetic nomenclature, including priority of publication, as a basis for naming homologues (Demerec *et al.*, 1966, *Genetics* **54:** 61–76). Although the same name might be used for different genes across subgroups, standardized names and the similar genetic organizations within the subgroups should greatly facilitate comparative studies and application of information learned in one species to the study of another.

As for the definition of the '*hrp*' designation, it now may include not only genes with a Hrp phenotype, but any gene associated with the Hrp pathway by function, homology, or location within a gene cluster or operon that is essential for the Hrp phenotype. We view use of the '*hrp*' designation in this larger sense as elective rather than mandatory. For example, the designation '*hpa*' has been used for Hrp-associated genes shown not to have a strict Hrp phenotype in *R. solanacearum* (Gough *et al.*, 1993, ibid.). In order to minimize confusion in the literature, we propose that this designation be maintained for such genes in this organism and in *X. campestris.* However, for *P. syringae* and the erwiniae, in which gene phenotypes may differ from species to species, we propose a unified nomenclature based on the more inclusive definition of *hrp* genes presented here. We hope that this broadened definition will help us to gain a focussed understanding of the key elements underlying the varied and intricate interactions of bacteria with plants.

For convenience, and because '*hrc*' represents a subset of *hrp* genes, *hrc* and *hrp* genes collectively will be referred to in general discussion as '*hrp*', as in the phrase 'the *hrp* genes of phytopathogenic bacteria.' The combined designation '*hrp/c*' may be used to specify a small group of genes, e.g. 'The genes are arranged co-linearly with their *hrp/c* homologues in *Xanthomonas campestris* pv. *vesicatoria*.' Operons containing *hrc* genes still may be referred to as '*hrp*' operons. When discussing homologues with the same name (*hrp* or *hrc*) from more than one plant pathogen, distinctions can be made where necessary using abbreviations for the names of the different bacteria subscripted to the gene name.

The unified nomenclature for conserved *hrp* genes will benefit research in several ways. It makes the known homologies among plant pathogens explicit. It provides for easy cross-reference to other systems, particulary that of *Yersinia* spp. It facilitates writing and speaking cogently about *hrp* genes. Finally, it transforms a previously confusing jumble of gene names into a well-ordered catalogue, which is an accessible reference not only for *hrp* researchers, but also for those studying other type III secretion systems.

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Regulation of *hrp* Genes and Type III Protein Secretion in *Erwinia amylovora* by HrpX/HrpY, a Novel Two-Component System, and HrpS

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Two novel regulatory components, *hrpX* and *hrpY*, of the *hrp* system of *Erwinia amylovora* were identified. The *hrpXY* operon is expressed in rich media, but its transcription is increased threefold by low pH, nutrient, and temperature levels—conditions that mimic the plant apoplast. *hrpXY* is autoregulated and directs the expression of *hrpL*; *hrpL*, in turn, activates transcription of other loci in the *hrp* gene cluster (Z.-M. Wei and S. V. Beer, J. Bacteriol. 177:6201-6210, 1995). The deduced amino -acid sequences of *hrpX* and *hrpY* are similar to bacterial two-component regulators including VsrA/VsrD of *Pseudomonas* (*Ralstonia*) *solanacearum*, DegS/DegU of *Bacillus subtilis*, and UhpB/UhpA and NarX/NarP, NarL of *Escherichia coli*. The N-terminal signal-input domain of HrpX contains PAS domain repeats. *hrpS*, located downstream of *hrpXY*, encodes a protein with homology to WtsA (HrpS) of *Erwinia* (*Pantoea*) *stewartii*, HrpR and HrpS of *Pseudomonas syringae*, and other σ^{54}-dependent, enhancer-binding proteins. Transcription of *hrpS* also is induced under conditions that mimic the plant apoplast. However, *hrpS* is not autoregulated, and its expression is not affected by *hrpXY*. When *hrpS* or *hrpL* were provided on multicopy plasmids, both *hrpX* and *hrpY* mutants recovered the ability to elicit the hypersensitive reaction in tobacco. This confirms that *hrpS* and *hrpL* are not epistatic to *hrpXY*. A model of the regulatory cascades leading to the induction of the *E. amylovora* type III system is proposed.

Additional keywords: fire blight, pathogenicity, virulence.

Erwinia amylovora is the causal agent of the fire blight disease of many rosaceous plants including pear and apple (van der Zwet and Beer 1999). The bacterium infects blossoms, leaves, succulent shoots, and immature fruits. Symptoms of the infected plants include water soaking and discoloration, followed by necrosis. Sometimes the disease kills whole trees or substantial portions, resulting in devastating economic loss. In nonhost plants such as tobacco and Arabidopsis, the bacterium elicits the defensive hypersensitive reaction (HR), which is characterized by rapid, localized, cell death (Goodman and Novacky 1994). For infection and HR induction, genes generally called *hrp* (hypersensitive response and pathogenicity; see Alfano and Collmer 1996 for a review) are essential.

The *hrp* gene cluster of *E. amylovora* Ea321 has been cloned in several cosmids and enables nonpathogenic bacteria such as *Escherichia coli* to elicit the HR in plants (Beer et al. 1991). According to phenotypic analyses of mutants, *hrp* genes of *E. amylovora* are localized within a 25-kb region of DNA, consisting of at least eight transcriptional units (Wei and Beer 1993). Sequence analysis (Bogdanove et al. 1996; Kim et al. 1997) indicated that the majority of *hrp* genes encode proteins that are thought to be components of a specialized protein secretion apparatus called the type III pathway (Hrp pathway for plant pathogens) (Galán and Bliska 1996). Several proteins including harpins (HrpN and HrpW) and a pathogenicity/avirulence protein (DspE) have been shown to be secreted via the pathway (Bogdanove et al. 1998a; Kim and Beer 1998; Wei and Beer 1993).

Transcriptional expression of *hrp* genes is induced under conditions similar to the environment of the plant apoplast: low carbon and nitrogen, low pH (5.5), and low temperature (18°C) (Wei et al. 1992). Two independent loci, complementation groups IV and V, in the *hrp* cluster were found to have regulatory function (Sneath et al. 1990; Wei and Beer 1993, 1995). Mutations in these loci abolish harpin production and the HR-eliciting and disease-causing abilities of *E. amylovora* (Wei and Beer 1993). Preliminary sequence analysis indicated that one of them (group IV) contains a gene called *hrpS* (Sneath et al. 1990) that encodes a protein similar to σ^{54}-dependent transcriptional activators (Morett and Segovia 1993). Complementation group V encodes *hrpL* (Wei and Beer 1995), which is homologous to genes encoding members of the ECF subfamily of eubacterial sigma factors (Lonetto et al. 1994). HrpL recognizes conserved promoter sequences called "*hrp* boxes" (Xiao and Hutcheson 1994), and directs the transcription of other pathogenicity genes including *hrp* secretion operons (*hrpA*, *hrpC*, and *hrpJ*) (Wei and Beer 1995), harpin genes (*hrpN* and *hrpW*) (Kim and Beer 1998; Wei and Beer 1995), and a disease-specific locus (*dspEF* [Bogdanove et al. 1998b]; *dspAB* [Gaudriault et al. 1997]).

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Nucleotide and/or amino acid sequence data have been deposited in the GenBank data base under accession number AF083877.

Here we report the characterization of two new regulatory genes, designated *hrpX* and *hrpY*, and the further analysis of *hrpS*. *hrpX* and *hrpY* are present in an operon situated between *hrpS* and *hrpL*. Analysis of deduced protein sequences suggested that they constitute a two-component regulatory complex; HrpX functioning as a sensor and HrpY as the response-regulator partner of HrpX. *hrpX*, *hrpY*, and *hrpS* are components of a complex regulatory network that leads to activation of *hrpL* and eventually other genes in the *hrp* cluster of *E. amylovora*.

RESULTS

Identification and sequence analysis of the *hrpXY* locus.

Previous studies have identified several loci, including *hrpC*, *hrpA*, *hrpS*, *hrpL*, and *hrpJ*, that are essential for the Hrp phenotype (Bogdanove et al. 1996; Kim et al. 1997; Wei and Beer 1993, 1995) (Fig. 1A). Preliminary genetic analysis of pCPP430 in *Escherichia coli* suggested the presence of a new locus, between *hrpS* and *hrpL*, that also is required for the Hrp phenotype and contains novel regulatory components. We have designated this locus *hrpXY*.

A 3.4-kb *Bgl*II- and *Cla*I-digested fragment of pCPP430 was cloned into pBluescript KS+, resulting in pCPP1178. The sequence of the insert of pCPP1178 revealed two tightly linked open reading frames (ORFs) between *hrpL* and *hrpS* that are capable of encoding proteins of 495 and 213 amino acid residues, respectively (Fig. 1B). These ORFs were named *hrpX* and *hrpY*, respectively. Potential ribosome-binding sites, AGGAG and TGGAA, were found 5 and 7 bp upstream of the *hrpX* and *hrpY* start codons, respectively. Although the ribosome-binding site ahead of *hrpY* weakly matches the consensus sequence, we assume it is sufficient for translation of *hrpY*; only a 4-bp space exists between the *hrpX* stop codon and *hrpY* start codon and translational coupling is plausible. To confirm that the *hrpX* and *hrpY* ORFs produce proteins, pCPP1178 was placed in a gene expression system mediated by the T7 RNA polymerase. Two distinct protein bands were visible following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The apparent molecular masses of HrpX and HrpY were about 50 and 25 kDa, respectively (data not shown), close to the sizes expected from the deduced amino acid sequences.

The start codon of *hrpX* is located 146 bp downstream of the *hrpL* stop codon, and a promoter prediction program (see Materials and Methods) identified two putative σ^{70} promoter sequences, TAGACG-N_{17}-TAAAGT (score from promoter prediction by neural network = 0.97) and TTGCAA-N_{16}-CCTAAT (score = 0.95), 111 and 33 bp upstream of the *hrpX* start codon, respectively. There is a 361-bp noncoding region between *hrpY* and *hrpS*. Palindromic sequences that may serve either as targets of regulatory components or as transcription terminators, GTAAACANTGTTTAC and GGATAAAATGG-TTGTGG-N_7-CCGCTTCCATTTTATCC, were identified in the *hrpL-hrpX* and *hrpY-hrpS* intergenic regions, respectively. The tight linkage of *hrpX* and *hrpY*, and the existence of long noncoding areas and inverted repeats upstream of *hrpX* and downstream of *hrpY*, suggest that the two genes form an operon.

HrpX and HrpY constitute a two-component regulatory system.

Comparison of the predicted amino acid sequences of *hrpX* and *hrpY* with sequences in the data bases revealed significant similarities with many two-component regulatory proteins. The homologs include VsrA/VsrD of *Pseudomonas* (now *Ralstonia*) *solanacearum*, which regulate virulence gene expression (Huang et al. 1995b); UhpB/UhpA of *Escherichia coli*, which participate in the regulation of sugar transport (Friedrich and Kadner 1987); NarX/NarP,NarL of *Escherichia*

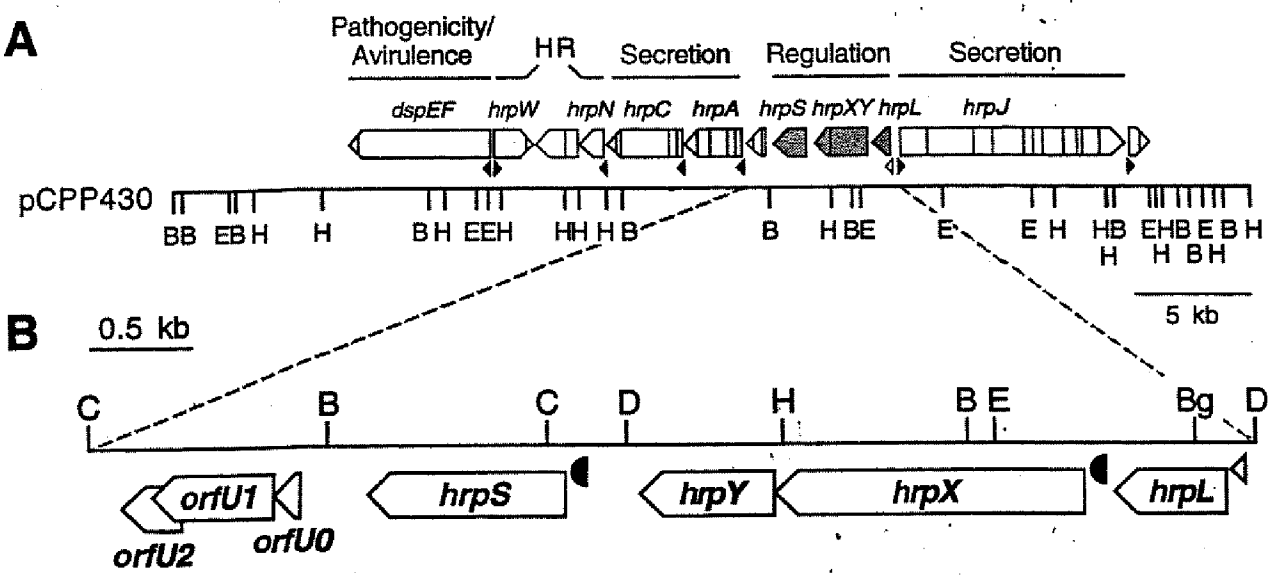

Fig. 1. A, Operon organization of the *hrp/dsp* gene cluster of *Erwinia amylovora* cloned in pCPP430. B, Central region covering regulatory genes *hrpL*, *hrpX*, *hrpY*, and *hrpS*. Boxes and arrow boxes: transcriptional units or open reading frames; names of the characterized operons or genes are given above, inside, or below. Filled triangles: putative HrpL-dependent promoters. Open triangles: putative σ^{54} promoters. Closed half circles: putative σ^{70} promoters. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Bg, *Bgl*II; C, *Cla*I; and D, *Dra*I.

coli, which are involved in the regulation of anaerobic respiratory gene expression (Rabin and Stewart 1993); and DegS/DegU of *Bacillus subtilis*, which are involved in extracellular enzyme production (Kunst et al. 1988) (Fig. 2; Table 1). In addition, HrpY showed high sequence similarity with many other transcriptional activators including ExpA of *E. carotovora* (33% identity), which is involved in global control of virulence (Eriksson et al. 1998); UvrY of *Escherichia coli* (33% identity) (Sharma et al. 1986); SirA of *Salmonella typhimurium* (32% identity) (Johnston et al. 1996); and GacA of several animal- and plant-associated *Pseudomonas* spp. (29 to 30% identities) (Laville et al. 1992).

The high sequence similarity of HrpX with histidine kinases suggests that HrpX is a sensor. HrpX has the conserved His

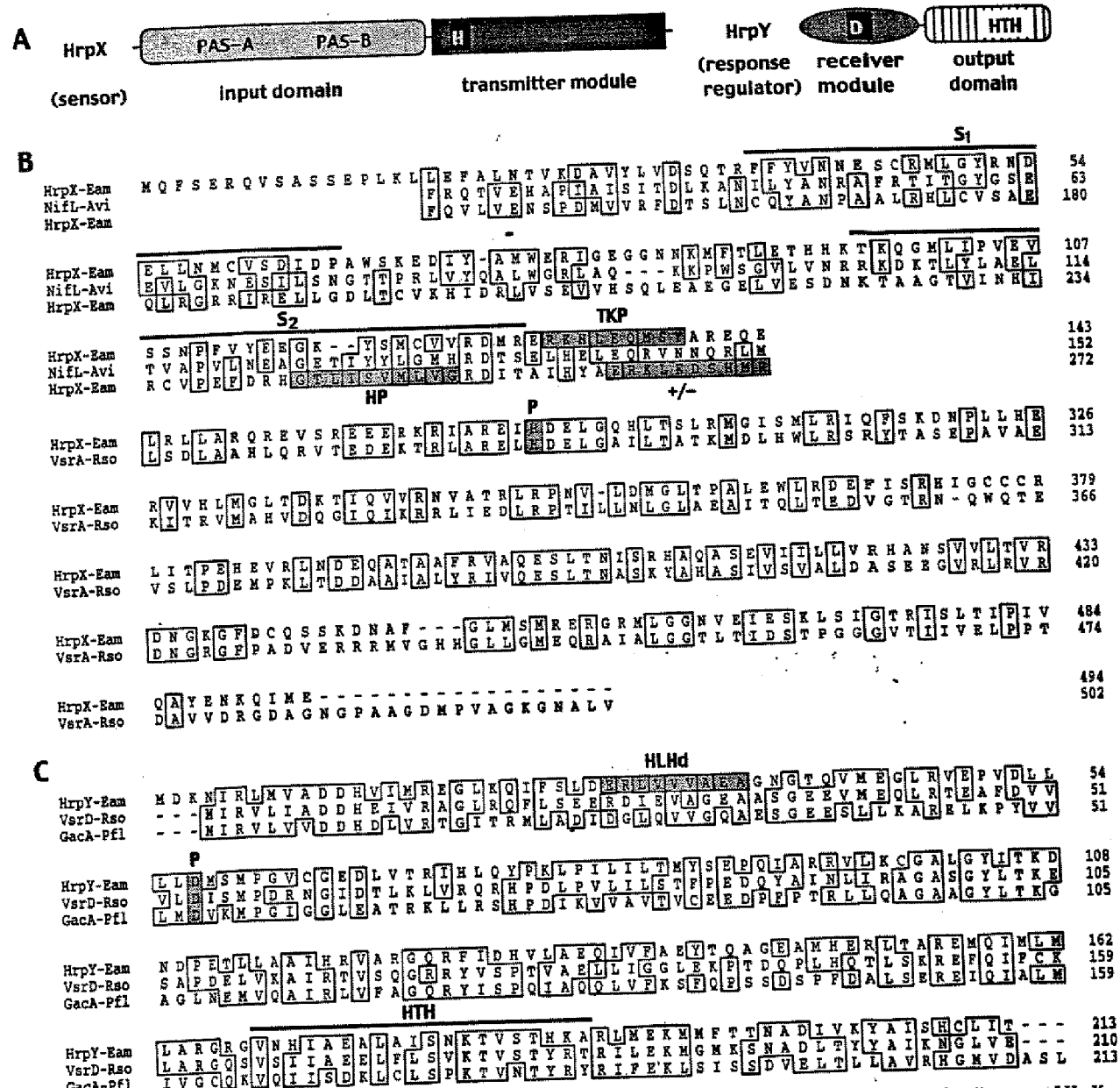

Fig. 2. A, Diagramatic illustrations of HrpX and HrpY of *Erwinia amylovora* showing predicted domain structures. **B and C,** Alignment of HrpX and HrpY with similar proteins. Designations of diagrams are after Parkinson and Kofoid (1992). PAS-A and PAS-B denote two repeats of the PAS domain, H and D phosphorylated histidine and aspartate residues, respectively, and HTH the helix-turn-helix DNA-binding motif. Overlines represent the S-boxes in the PAS domain (S_1 and S_2) and the HTH motif. A putative tyrosine kinase phosphorylation site (TKP), a hydrophobic region (HP), a putative charge-rich linker (+/–), and a helix-loop-helix dimerization domain signature (HLHd), and phosphorylation sites (P) are shown by shading. PILEUP program (GCG software package, vers. 7.3; Genetics Computer Group, Madison, WI, U.S.A.) with default parameters was used to align the sequences. Accession numbers of the compared proteins: NifL of *Azotobacter vinelandii*, SWISS-PROT:P30663; VsrA and VsrD of *Ralstonia solanacearum*, PIR:S41544 and PIR:I40540; and GacA of *Pseudomonas fluorescens*, SWISS-PROT:P32967. In the HrpX alignment, only 27 to 152 residues of NifL and C-terminal 243 residues of VsrA are shown. Also, the HrpX sequence is shown on two lines for the first three rows.

residue for autophosphorylation and a hydrophobic domain that may enable the protein to be transiently associated with the cytoplasmic membrane (Fig. 2B). The C-terminal putative transmitter domain (residues 273 to 494) of HrpX shows most similarity to the kinase domains of the sensor proteins listed in Table 1; the N-terminal putative input domain of HrpX shows similarity to PAS domains (Zhulin et al. 1997) of *Methanobacterium thermoautotrophicum*, *Azotobacter vinelandii*, and other organisms. Several PAS-containing proteins are sensors of bacterial two-component systems. The PAS domain typically consists of two direct sequence repeats (PAS-A and PAS-B), and each repeat contains two highly conserved regions called S_1 and S_2 boxes (Zhulin et al. 1997). In the case of HrpX, the second repeat (PAS-B) seems imperfect (Fig. 2B). Based on ScanProsite analysis (Appel et al. 1994), another feature of HrpX with unknown functional relevance is a putative tyrosine kinase phosphorylation site (PROSITE:PS00007).

HrpY appears to be a response regulator with a putative receiver domain at the N terminus (up to 102 amino acid residues) and a DNA-binding domain at the C terminus (Fig. 2A). As shown in Figure 2C, HrpY contains the conserved Asp residue, which may be phosphorylated by the sensor, and the helix-turn-helix DNA binding motif. HrpY also has a sequence that matches the Myc-type helix-loop-helix dimerization domain signature (PROSITE:PS00038), the functional significance of which remains to be tested.

Table 1. HrpX and HrpY of *Erwinia amylovora* compared with two-component regulatory proteins (sensors/response regulators) of other bacteria

Bacterium	Protein	Amino acids	% Identity[a]
Erwinia amylovora	HrpX/HrpY	494/213	–
Ralstonia solanacearum	VsrA/VsrD	502/210	34/41
Escherichia coli	UhpB/UhpA	500/196	32/32
Bacillus subtilis	DegS/DegU	385/229	32/28
Escherichia coli	NarX/NarP, NarL	598/215, 216	31/33, 32

[a] % Identities from a BLASTP search of HrpX and HrpY with default parameters, except for no filtering for low complexicity regions. Only the transmitter domain of HrpX (residues 273 to 494) was used for comparisons with other sensor proteins.

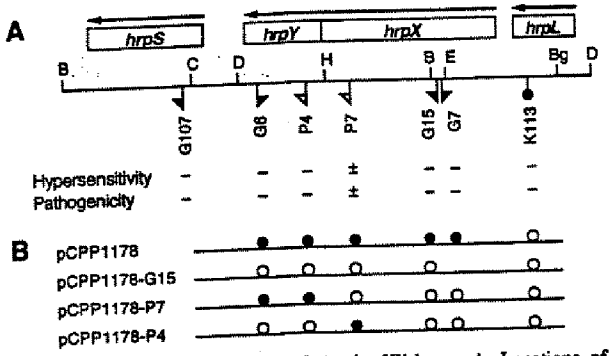

Fig. 3. Genetic characterization of the *hrpXY* locus. **A**, Locations of transposon insertions and phenotypes of *hrpX* and *hrpY* mutants of *Erwinia amylovora* Ea321. Rectangles above map of restriction enzymes and transposons represent transcriptional units. Arrows: directions of transcription. Closed flags: insertions by Tn*5-gusA1*. Open flags: insertions by Tn*phoA*. Llollipop: a Tn*10*-miniKm insertion. Mutants shown by minus signs below insertion points did not elicit the hypersensitve reaction (HR) or cause disease (Hrp$^-$); a mutant shown by ± infrequently elicited spotty HR and showed low virulence. **B**, Complementation assay of *hrpX* and *hrpY* mutants of *E. amylovora* Ea321 with various plasmids. Closed circle: plasmid complemented Hrp phenotype of the mutant containing the transposon insertion in the same column. Open circle: plasmid did not change the phenotype of corresponding mutant.

Genetic characterization of *hrpX* and *hrpY*.

The *hrpXY* locus in pCPP430 was mutagenized with transposons Tn*5-gusA1* and Tn*phoA*. Derivatives of pCPP430 containing the transposon insertions were marker-exchanged into the genome of *E. amylovora* Ea321. All *hrpY* mutants of Ea321 failed to elicit the HR in tobacco and to infect immature pear fruits (Fig. 3A). Two classes of *hrpX* insertion mutants were obtained. Ea321-G15 and Ea321-G7, which were made with Tn*5-gusA1*, were similar to *hrpY* mutants in phenotypes. Ea321-P7, an *hrpX*::Tn*phoA* mutant, caused slight tissue collapse in tobacco at higher inoculum dose and had low virulence in immature pears, rather than the strict Hrp$^-$ phenotype (Fig. 3A). Specifically, tobacco leaves infiltrated with Ea321-P7 at $\geq 5 \times 10^8$ CFU per ml developed a spotty HR 36 h after infiltration. Also, in immature pears inoculated with the mutant, bacterial ooze appeared 3 days later than in those inoculated with the wild type, and the population of the mutant recovered was only one-tenth of that of the wild type (data not shown).

Virulence of the mutants was restored to near wild-type levels by providing the mutants with pCPP1178 in *trans* (Fig. 3B). The *hrpX*::Tn*5-gusA1* mutants of Ea321 were not complemented by pCPP1178-P4 that contains a transposon insertion in *hrpY* (Fig. 3B). This suggests that *hrpX* and *hrpY* are in the same transcriptional unit and the Tn*5-gusA1* mutations in *hrpX* are polar. We found, however, that the *hrpX*::Tn*phoA* mutant Ea321-P7 can be complemented by pCPP1178-P4, indicating that the Tn*phoA* insertion of *hrpX* did not affect the function of *hrpY* (Fig. 3B). Tn*phoA*-induced mutations that permit the expression of downstream genes have been observed frequently in *E. amylovora* (Z. Wei and S. V. Beer, *unpublished data*) and *Pseudomonas syringae* (Huang et al. 1995a). Thus, we believe that the P7 insertion is nonpolar and that the peculiar phenotype of the Ea321-P7 may reflect the function of *hrpX*.

All the transposon mutations in the *hrpXY* locus were complemented by derivatives of pCPP430 with transposon insertions in *hrpS* or *hrpL* (data not shown), confirming the suggestion from sequence analysis that *hrpX* and *hrpY* constitute an independent complementation group. Based on results of sequence analysis and genetic characterization, we conclude (i) *hrpXY* is required for the Hrp phenotype, and (ii) *hrpX* and *hrpY* constitute a two-gene operon, *hrpXY*.

Expression of *hrpXY* is environmentally regulated.

A new construct, pCPP1203, was used to monitor expression of the *hrpXY* promoter in a nutrient-rich medium and a minimal medium that induces the expression of *hrp* genes (Wei et al. 1992). pCPP1203 was derived from pCPP1178-G15 (*hrpX*::Tn*5-gusA1*) in which the directions of *hrpX* and *gusA* are the same. pCPP1178-G15 was digested with *Bam*HI and *Sac*I (an *Sac*I site is present in the vector), which cuts out the *hrpXY* promoter region, a 5′ portion of the *hrpX* coding region fused to Tn*5-gusA1*, and the whole transposon. The resulting fragment was then ligated to pCPP43, which had been digested with the same enzymes. pCPP43 (gift of David

W. Bauer) is a derivative of pOU61, which is a low-copy-number plasmid (approximately one copy per bacterium at 30°C) (Larsen et al. 1984).

In *E. amylovora* and *Escherichia coli*, the *hrpXY* promoter directed high levels of basal expression in Luria broth (LB), but expression of *hrpX*::Tn*5-gusA1* was enhanced threefold in the *hrp*-inducing minimal medium (IM) (Table 2). Enhanced levels of *hrpX*::Tn*5-gusA1* expression were also observed from assays of the strains in tobacco leaves and immature pears (data not shown). No β-glucuronidase (GUS) activity was detected for *Escherichia coli* SØ200Δ*uidA*(pCPP1203) unless functional *hrpXY* was provided (Table 2). Similarly, high basal-level expression of *hrpX*::Tn*5-gusA1* of Ea321(pCPP1203) in Table 2 is probably due to functional *hrpXY* present in the chromosome. The latter two observations indicate that *hrpXY* is also autoregulated.

hrpX and *hrpY* control the expression of *hrpL*.

To study the effect of *hrpX* and *hrpY* on the control of *hrpL* expression, a *hrpL*::Tn*5-gusA1* fusion (pCPP139-G44) (Wei and Beer 1995) was marker exchanged into an *hrpX* mutant (Ea321-P7) and an *hrpY* mutant (Ea321-P4), to generate *hrpX-hrpL* and *hrpY-hrpL* double mutants Ea321-P7G44 and Ea321-P4G44, respectively. Mutation in *hrpY* completely abolished *hrpL* expression (Fig. 4). However, the *hrpX* mutant reduced *hrpL* expression only to about 20% of its wild-type level, opening the possibility that the mutated HrpX may be still partially functional or another sensor protein may cross talk with HrpY.

Analysis of the *hrpS* locus and the ORFs between *hrpS* and *hrpA*.

hrpS also partially controls *hrpL* expression in *E. amylovora* and is located downstream of *hrpXY* (Wei and Beer 1995). We report here the entire nucleotide sequence of the region between *hrpY* and *hrpA*, which includes *hrpS*, to complete the preliminary results on *hrpS* presented previously (Sneath et al. 1990).

The *hrpS* locus of *E. amylovora* Ea321 contains a single-gene operon, based on the large intergenic regions beyond the coding region of *hrpS*, and a potential terminator, CGGCGACAGC-N_8-GCTGTCGCCG, that lies 49 bp downstream of the *hrpS* stop codon. The *hrpS* ORF is preceded by a potential σ^{70} promoter, GTGGCA-N_{18}-TATTAC (score from promoter prediction by neural network = 0.96), and it encodes a 324 amino acid protein. HrpS has homology to members of the σ^{54}-dependent, enhancer-binding protein family (Morett and Segovia 1993). HrpS shows highest sequence similarity with WtsA (HrpS) of *Erwinia* (*Pantoea*) *stewartii* (Frederick et al. 1993) (79% identity over 322 amino acid residues without gaps from BLASTP), HrpR and HrpS of *P. syringae* pathovars (51 to 55% identities) (Grimm et al. 1995; Xiao et al. 1994), and DctD of *Rhizobium* spp. (39% identities) (Jiang et al. 1989; Ronson et al. 1987). HrpS of *E. amylovora* has two putative ATP-binding sites at the N terminus and a helix-turn-helix DNA-binding motif at the C terminus (Fig. 5A). HrpS shows high sequence similarity to other regulators in the NtrC family throughout the entire σ^{54} interaction domain. However, similar to other HrpR/HrpS proteins, HrpS of *E. amylovora* contains a very short N-terminal A domain (Shingler 1996), and seems to lack the phosphorylation receiver domain (Fig. 5A).

In the region between *hrpS* and *hrpA*, three potential genes, designated *orfU0*, *orfU1*, and *orfU2* (Fig. 1B), were identified by application of the GeneMark.hmm algorithm (Lukashin and Borodovsky 1998). *orfU0* is a small ORF encoding a 46 amino acid basic protein, without significant similarity to any protein in the data base. Preceded by GGAGT 8 bp upstream, *orfU1* encodes a 203 amino acid basic protein that is similar to a conserved hypothetical protein HP1401 of *Helicobacter pylori* (32% identity over 164 amino acid residues with 12 gaps) (Fig. 5B). Interestingly, protein sequence of the next ORF, *orfU2*, shows even higher similarity to HP1401 (residues 189 to 229; 41% identity without gaps). This suggests the possibility that a frame shift in *orfU1-orfU2* resulted in the two current ORFs, and that both may be defective. The lack of an obvious promoter in front of *orfU0*, the lack of good ribosome-binding sites in front of *orfU0* and *orfU2*, the potential frame-shift mutation at the 3′ region of *orfU1*, and the lack of a phenotype of Tn*phoA*-induced *orfU1* mutants (data not shown) indicate that the region comprising *orfU0-orfU2* is unlikely to be functional in Ea321.

Expression of *hrpS* is not autoregulated, and induction of *hrpS* is independent of *hrpX* or *hrpY*.

An *hrpS*::*gusA1* fusion designated G107 (Wei et al. 1992) was used to assay the expression of *hrpS*. A fragment of

Table 2. Expression of the *hrpXY* promoter in Luria broth (LB) and in a *hrp*-inducing minimal medium (IM)

Bacterial strain[a]	GUS activity[b]	
	LB	IM
Erwinia amylovora Ea321(pCPP1203)	242 ± 12	788 ± 32
E. coli SØ200Δ*uidA*(pCPP1203)	2 ± 3	3 ± 3
E. coli SØ200Δ*uidA*(pCPP1203, pCPP1178)	145 ± 19	878 ± 33

[a] *E. coli* SØ200Δ*uidA* is an *Escherichia coli* strain with no β-glucuronidase (GUS) activity due to deletion of *gusA*. pCPP1203 is a low-copy-number plasmid containing *hrpX*::Tn*5-gusA1*; pCPP1178 is a high-copy-number plasmid containing functional *hrpX* and *hrpY* genes.
[b] Picounits per CFU; mean of three replicates ± standard deviation.

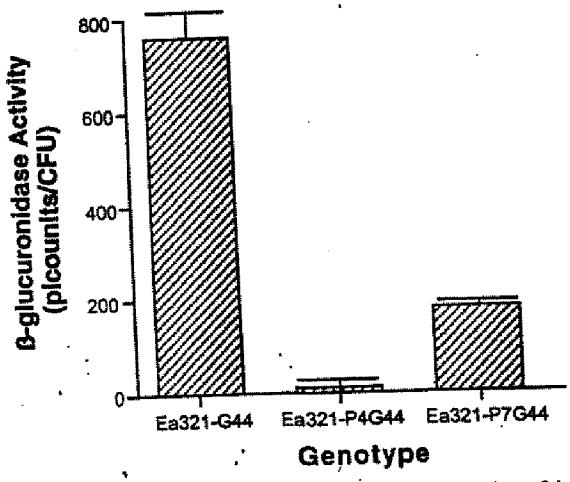

Fig. 4. Effect of mutations in *hrpX* and *hrpY* on expression of *hrpL*. Genotypes of the strains are Ea321-G44, *hrpL*::Tn*5-gusA1* (Wei and Beer 1995); Ea321-P4G44, *hrpY*::Tn*phoA* and *hrpL*::Tn*5-gusA1*; and Ea321-P7G44, *hrpX*::Tn*phoA* and *hrpL*::Tn*5-gusA1*. Error bars: standard deviation from three replicates. Cells grown in inducing medium (IM) were assayed (see Materials and Methods for details).

pCPP430-G107 digested with *Bam*HI contains the whole transposon, the *hrpS* gene fused to Tn*5-gusA1*, and the *hrpS* promoter region. This *Bam*HI fragment was ligated with a low-copy-number plasmid, pCPP8 (Bauer 1990), that was cut with the same enzyme. The resulting plasmid was designated pCPP1058. As with *hrpXY*, expression of *hrpS* in *Escherichia coli* or in *E. amylovora* was induced under *hrp*-inducing conditions (Table 3). However, autoregulation was not required for *hrpS* expression; the presence of functional *hrpS* did not affect the expression of a *hrpS*::*gusA1* fusion in pCPP1058 (Table 3).

To determine whether the newly discovered two-component system has any effect on the expression of *hrpS*, an *hrpS*::Tn*5-gusA1* fusion (pCPP430-G107) was marker-exchanged into *hrpX* and *hrpY* mutants. Neither *hrpX* nor *hrpY* affected *hrpS* expression significantly (Fig. 6).

hrpS and _hrpL_, provided by multicopy plasmids, suppress defects in _hrpX_ or _hrpY_.

To further characterize the regulatory relationships between *hrpXY*, *hrpS*, and *hrpL*, the HR-impaired strains Ea321-P7, Ea321-P4, and Ea321-G107 were transformed with pCPP1178

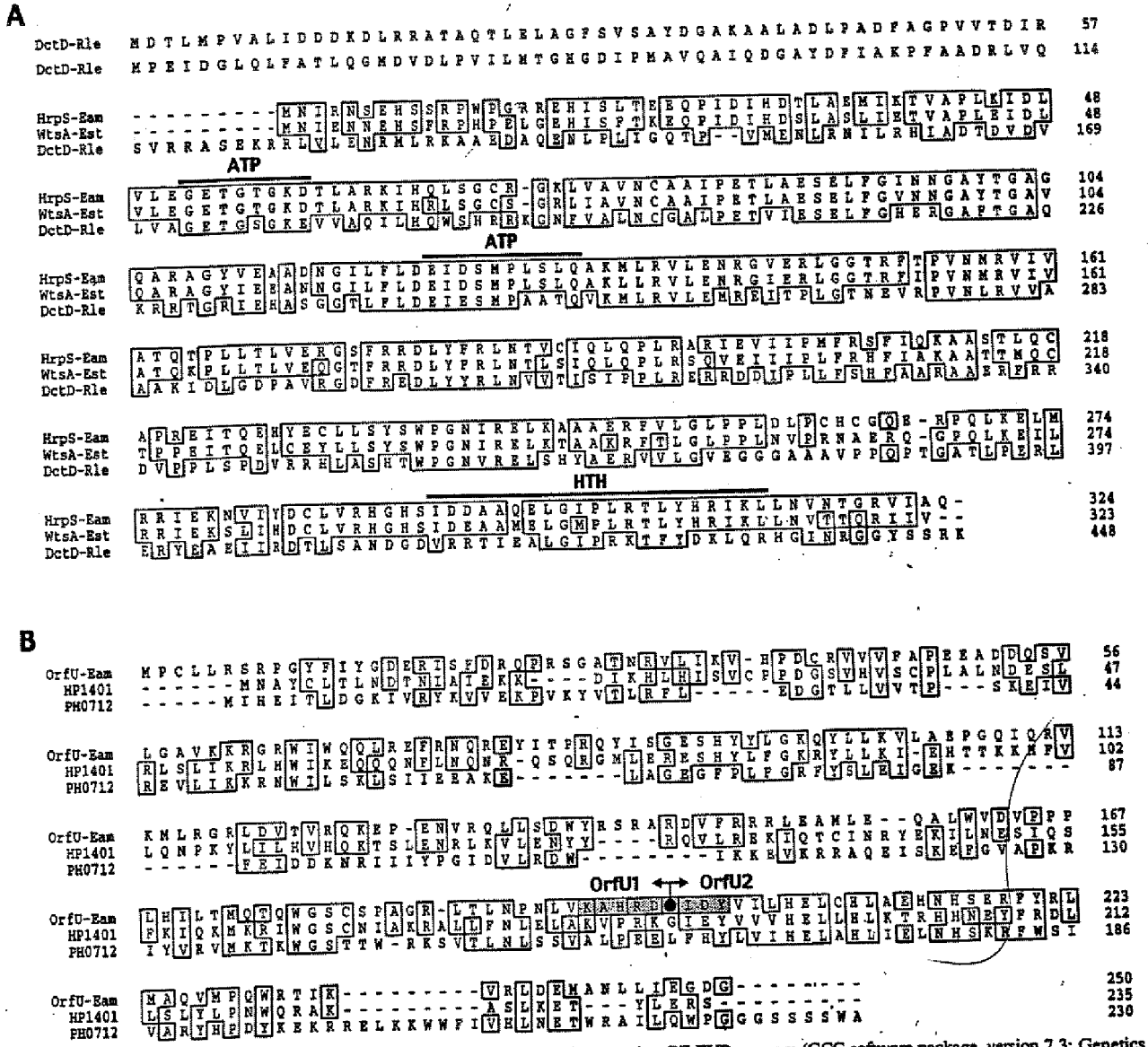

Fig. 5. Alignments of **A**, HrpS, and **B**, OrfU of *Erwinia amylovora* with similar proteins. PILEUP program (GCG software package, version 7.3; Genetics Computer Group, Madison, WI, U.S.A.) with default parameters was used to align the sequences. Overlines represent ATP-binding sites (ATP) and the helix-turn-helix DNA-binding motif (HTH). Sequence of OrfU is a composite of sequences of *orfU1* and *orfU2* products. A putative tyrosine kinase phosphorylation site (PROSITE: PS00007) is indicated by shading. Black circle in the OrfU sequence denotes location of a probable reading-frame shift. Accession numbers: WtsA of *E. stewartii*, SWISS-PROT:P36219; DctD of *Rhizobium leguminosarium*, SWISS-PROT:P10046; HP1401 of *Helicobacter pylori*, GENBANK:AE000640; and PH0712 of *Pyrococcus horikoshii*, DDBJ:AP000003.

(contains *hrpXY*), pCPP1001 (contains *hrpS*) (Wei and Beer 1995), or pCPP1078 (contains *hrpL*) (Wei and Beer 1995). The resulting transformants were infiltrated into panels of tobacco leaves to determine which, if any, of the regulatory genes, when present in multiple copies, are sufficient to restore the HR-eliciting ability to the mutants. Panels infiltrated with *hrpX* and *hrpY* mutants containing *hrpL* developed the HR (Table 4), often faster than panels infiltrated with the wild-type strain. The panels began to show collapse 8 to 12 h after infiltration; by 24 h, the whole infiltrated area had collapsed in a typical HR. This result is consistent with dependence of *hrpL* expression on *hrpX* and *hrpY*. Interestingly, similar suppression was observed from *hrpX* and *hrpY* mutants containing *hrpS*, whereas *hrpX* and *hrpY* did not restore the HR phenotype of the *hrpS* mutant (Table 4).

Table 3. Expression of the *hrpS* promoter in Luria broth (LB) and in *hrp*-inducing minimal medium (IM)

Bacterial strain[a]	GUS activity[b]	
	LB	IM
E. coli SØ200Δ*uidA*(pCPP1058)	94 ± 12	367 ± 9
E. coli SØ200Δ*uidA*(pCPP1058, pCPP1001)	105 ± 17	378 ± 23
Erwinia amylovora Ea321-G107	36 ± 11	188 ± 35
Erwinia amylovora Ea321-G107(pCPP1001)	42 ± 21	229 ± 29

[a] *E. coli* SØ200Δ*uidA* is an *Escherichia coli* strain with no β-glucuronidase (GUS) activity due to deletion of *gusA*. *Erwinia amylovora* Ea321-G107 is a mutant of Ea321 containing a Tn*5-gusA1* insertion in *hrpS* (Wei et al. 1992). pCPP1058 is a low-copy-number plasmid containing *hrpX*::Tn*5-gusA1*; pCPP1001 is a high-copy-number plasmid containing the functional *hrpS* gene and its promoter (Wei and Beer 1995).

[b] Picounits per CFU; meanof three replicates ± standard deviation.

DISCUSSION

The HrpX/HrpY two-component protein system.

Our results demonstrate that *E. amylovora* employs the HrpX/HrpY two-component regulatory proteins to direct expression of an alternate sigma factor gene, *hrpL*, that in turn activates a type III protein secretion system. This provides for a quick change in the pattern of gene expression needed to initiate infection. HrpX is a putative I_cT-type sensor (Parkinson and Kofoid 1992) composed of the N-terminal PAS domain and the C-terminal histidine kinase domain (Fig. 2A). HrpX appears to be cytoplasmic, and may be anchored to the inner membrane by its internal hydrophobic region. Other members of the PAS-containing I_cT-type sensor kinases include NifL, NtrB, and KinA (Zhulin et al. 1997). HrpY appears to be a RO_{III} subfamily response regulator (Parkinson and Kofoid 1992). Consistent with the HrpX transmitter domain, HrpY shows significant sequence similarity to VsrD, DegU, UhpA, and NarL.

Two-component systems with PAS domains in the sensor component include NifL/NifA, DctS/DctR, and BvgS/BvgA (Zhulin et al. 1997). Among these only NifL does not contain the periplasmic domain, and HrpX is more similar to NifL than the other two. NifL and most other PAS-containing proteins are sensors (Zhulin et al. 1997), and their signal input domains are located at the N terminus (Parkinson and Kofoid 1992). Thus, HrpX may directly perceive environmental signals with its N-terminal PAS domain. One function of the PAS domain is to act as a protein dimerization motif (Kay 1997). This raises the possibility of HrpX dimerization, which is required for the functional state of two-component sensors (Parkinson and Kofoid 1992).

Two-component regulatory system and type III protein secretion.

Although the two-component system is widely used to control bacterial gene expression (Hoch and Silhavy 1995), reports of its function in regulation of the type III system are just emerging. In *S. typhimurium*, SirA is a response regulator essential for induction of *hilA*, *prgHIJK*, and *sigDE* (Hong and Miller 1998; Johnston et al. 1996), and the PhoQ/PhoP two-component system represses the expression of the *prg* locus (Pegues et al. 1995). The CpxA/CpxR system controls the pH-dependent expression of the *Shigella sonnei virF* gene, which in turn activates *ipaBCD* and *virG* (Nakayama and Watanabe

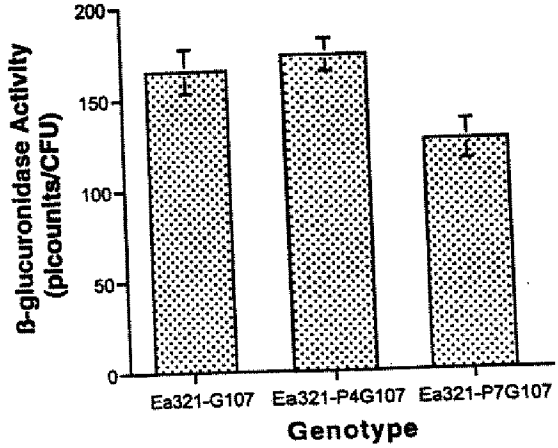

Fig. 6. Effect of mutations in *hrpX* and *hrpY* on expression of *hrpS*. Genotypes of the strains are Ea321-G107, *hrpS*::Tn*5-gusA1* (Wei et al. 1992); Ea321-P4G107, *hrpY*::Tn*phoA* and *hrpS*::Tn*5-gusA1*; and Ea321-P7G107, *hrpX*::Tn*phoA* and *hrpS*::Tn*5-gusA1*. Error bars: standard deviation for three replicates. Cells grown in inducing medium (IM) were assayed (Materials and Methods contains details).

Table 4. Hypersensitive reaction (HR) elicitation by *hrp* regulation mutants

Strain	Genotype	HR phenotype[a]
Ea321	wild type; *hrp*$^+$	+++
Ea321-P7	*hrpX*	±
Ea321-P7(pCPP1178)	*hrpX*(*hrpXY*$^+$)	++[b]
Ea321-P7(pCPP1001)	*hrpX*(*hrpS*$^+$)	+++
Ea321-P7(pCPP1078)	*hrpX*(*hrpL*$^+$)	+++
Ea321-P4	*hrpY*	–
Ea321-P4(pCPP1178)	*hrpY*(*hrpXY*$^+$)	++[b]
Ea321-P4(pCPP1001)	*hrpY*(*hrpS*$^+$)	+++
Ea321-P4(pCPP1078)	*hrpY*(*hrpL*$^+$)	+++
Ea321-G107	*hrpS*	–
Ea321-G107(pCPP1178)	*hrpS*(*hrpXY*$^+$)	–
Ea321-G107(pCPP1001)	*hrpS*(*hrpS*$^+$)	+++

[a] +++, full HR manifested by complete tissue collapse throughout infiltrated area; ++, reduced HR, which is spotty and often coalescing; ±, infrequent collapse and small spotty necreosis for HR-positive leaves; and –, no HR. Inoculum concentration was approximately 2×10^8 CFU per ml. Ratings (consensus of four plants) were made 36 h after inoculation.

[b] Full HR was observed at inoculum levels of $\geq 5 \times 10^8$ CFU per ml.

1995). Also, the BvgS/BvgA system was recently found to be involved in the regulation of the type III secretion in *Bordetella bronchiseptica* (Yuk et al. 1998). Among plant pathogens, HrpG of *Xanthomonas campestris* pv. *vesicatoria*, a homolog of response regulators, has been shown to regulate *hrpXv* and *hrpA* expression (Wengelnik et al. 1996).

The structure of the input domain of *E. amylovora* HrpX appears to be exceptional, compared with sensor proteins involved in other type III systems, which contain two transmembrane regions and a periplasmic domain. The closest homologs of *E. amylovora* HrpY are SirA and BvgA, both of which are RO_{III}-type regulators (Parkinson and Kofoid 1992), whereas *X. campestris* HrpG belongs to the RO_{II} type, which includes *Escherichia coli* CpxR and OmpR, *S. typhimurium* PhoP, and *Agrobacterium tumefaciens* VirG. Thus, at least two types of transmitter-receiver systems appear to have evolved for control of type III systems in response to environmental stimuli in hosts. Also, the two two-component systems identified in the plant pathogens *E. amylovora* and *X. campestris* fall into different communication groups.

HrpS and mechanism of gene regulation.

HrpS is a member of the σ^{54}-dependent, enhancer-binding protein family. Both *hrpS* and *rpoN* are required for transcription of *hrp* genes in *P. syringae* pathovars (Grimm et al. 1995; Xiao et al. 1994). WtsA (HrpS) of *E. stewartii* controls expression of *wtsB*, which also requires the presence of σ^{54} (Frederick et al. 1993). In *E. amylovora*, HrpS partially regulates *hrpL* expression (Wei and Beer 1995), and a sequence, TGGCAC-N_5-TTGC, that perfectly matches the –24/–12 promoter consensus sequence is found at the promoter region of *E. amylovora hrpL*. The *hrpS* gene of *E. amylovora*, but not *hrpS* of *P. syringae* pv. *phaseolicola*, can complement the *hrpS* mutation in *E. stewartii* (Frederick et al. 1993). The HrpS sequences of the two erwinias are highly similar, and even the upstream noncoding regions appear to be conserved, except for the insertion of a 484-bp sequence, reminiscent of an IS (insertion sequence) element, 23-bp upstream of the *E. stewartii hrpS* ORF.

As a member of the NtrC family, HrpS is unusual in that it lacks a long N-terminal receiver domain. Control of protein activation by phosphorylation, by protein-protein interaction, and by signal molecule have been suggested for σ^{54}-dependent proteins (Shingler 1996). In the direct activation model, derepression by effectors seems to be a mechanism of protein activation. For DctD, DmpR, and XylR, deletion of the receiver domain results in constitutive activation of the proteins, suggesting that the receiver domain has a repressor function

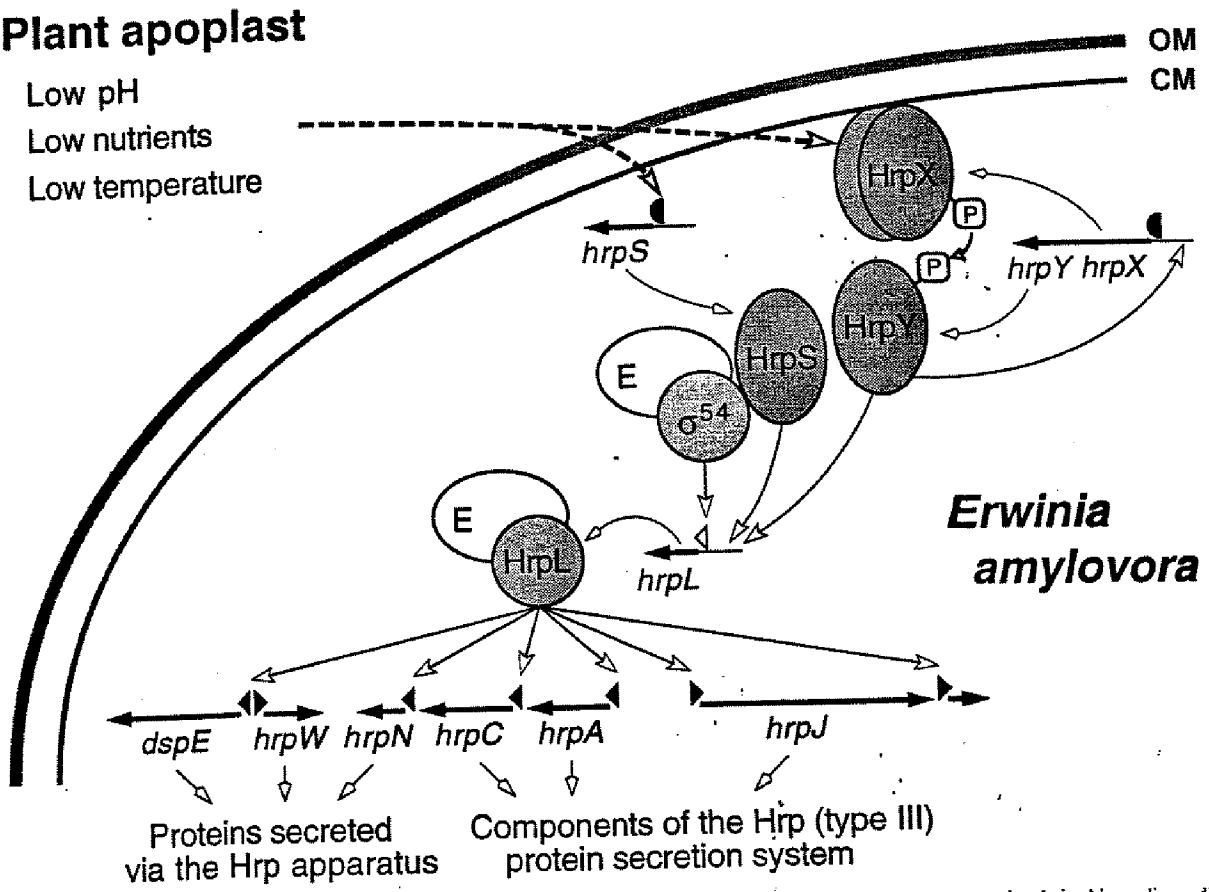

Fig. 7. Model of the *hrp* gene regulatory cascade. Thick arrow lines: genes or operons. Ovals and circles: proteins. Arrowheads in thinner lines: directions of information flow. CM, cytoplasmic membrane; OM, outer membrane; P, phosphate; E, RNA polymerase; closed half circle, σ^{70} promoter; open triangle, σ^{54} promoter; and filled triangle, HrpL promoter.

(Shingler 1996). Therefore, the apparent absence of the receiver domain in HrpS implies that HrpS may not require phosphorylation for activation and is always active once the protein is made.

Induction of *hrpXY* and *hrpS* and the involvement of HrpXY and HrpS in *hrpL* regulation.

Expression of *hrpS* and *hrpXY* is induced by conditions that mimic the apoplastic environment (Wei et al. 1992; this work). *hrpXY* shows high basal-level expression, and autoregulation is involved in gene induction. However, *hrpS* is not autoregulated based on results of the GUS assay, suggesting that there may be upstream regulatory components. Although *hrpS* provided in multiple-copy plasmids reverses the Hrp$^-$ phenotype of *hrpX* and *hrpY* mutants, the independence of *hrpS* from *hrpX* and *hrpY* suggests that *hrpXY* is not epistatic to *hrpS* and environmental signals may go to *hrpS* through a different pathway.

Earlier work on *hrpL* and *hrpS* (Wei and Beer 1995) established that HrpS partially controls *hrpL* expression. Our current work indicates that the HrpX/HrpY system contributes to *hrpL* induction. Based on the role of *hrpXY* and *hrpS* in regulating *hrpL* and the lack of effect of *hrpX* and *hrpY* in *hrpS* expression, one might place *hrpS* upstream of *hrpXY*. This notion is precluded, however, because *hrpXY* does not override *hrpS* mutation. As mentioned above, the opposite is not likely, either. Therefore, it seems that signals independently perceived by *hrpXY* and *hrpS* converge at *hrpL*.

Neither HrpS nor HrpY alone induce high levels of *hrpL* expression, suggesting that cooperation of HrpY and HrpS, possibly through protein-protein interaction, may be needed for full activation of *hrpL*. In this model, HrpS may be a positive activator of *hrpL*, while HrpX/HrpY may act as a modulator of *hrpL* transcription. Complementation of *hrpX* and *hrpY* mutants for the HR phenotype by overexpressed *hrpS* supports this model. The regulation of *eps* genes of *R. solanacearum* seems similar; both VsrD and PhcA regulators bind to the *xpsR* promoter region and control *xpsR* expression (Huang et al. 1995b). In *P. syringae* pv. *syringae*, HrpR and HrpS have been proposed to work together to control *hrpL* expression (Xiao et al. 1994), although a different opinion exists for homologous proteins in *P. syringae* pv. *phaseolicola* (Grimm et al. 1995).

***hrp* gene regulation and Hrp phenotypes.**

hrpY and *hrpS* seem to be crucial to the pathogenic life-style of *E. amylovora*, since their inactivation by mutagenesis results in loss of pathogenicity in immature pears (Wei et al. 1992; this work). The *hrpX* mutant, however, shows an attenuated phenotype: slightly lowered *hrpL* expression and reduced HR and virulence at higher inoculum doses. Currently, we cannot rule out the possibility of partial HrpX function in that mutant, even though leaky phenotypes of sensor mutants have been documented for other two-component systems (Stock et al. 1989). It is interesting to note that, although *hrpX* and *hrpS* mutants show different phenotypes (the former reduced Hrp and the latter Hrp$^-$), both are similarly affected in *hrpL* expression, i.e., only three- to fourfold reduction. This suggests that either there is a threshold level of *hrp* gene expression required for causing disease, or *hrpS* is involved in expression of other genes that contribute to pathogenicity. Further study might distinguish between these two possibilities.

The incomplete complementation of *hrpX* and *hrpY* mutants by *hrpXY* provided in a multicopy plasmid at lower inoculum levels ($\leq 2 \times 10^8$ CFU per ml) is intriguing and deserves further investigation. One explanation for the results could be that defective HrpX and HrpY in the mutants interact with functional HrpX and HrpY, and, possibly by forming heterodimers, interfere with the full activity. Alternatively, overproduced HrpX and HrpY may somehow down-regulate *hrpS* expression.

Model of the *E. amylovora hrp* gene expression.

Based on previous studies (Bogdanove et al. 1996, 1998b; Kim and Beer 1998; Kim et al. 1997; Wei and Beer 1995; Wei et al. 1992) and results described in this work, we propose a scheme of *hrp* gene regulation in *E. amylovora* (Fig. 7). When the bacteria enter the plant apoplast, HrpX perceives environmental signals and is phosphorylated. Activated HrpX then phosphorylates HrpY to activate it, and increases the expression of *hrpXY* to produce more HrpX and HrpY. Independently, expression of *hrpS* is induced in response to the changed environment. Activated HrpY and HrpS, bound to the *hrpL* promoter, then interact with the RNA polymerase-σ^{54} complex to drive transcription of *hrpL*. HrpS also activates other genes containing the –24/–12 promoter consensus sequence. Finally, the HrpL σ factor, which recognizes a conserved promoter motif, GGAACC-N_{15}-CCACTAAT, directs transcription of the remaining *hrp* and *dsp* genes that produce the secretion machinery and virulence proteins that interact with plant cells.

MATERIALS AND METHODS

Bacterial strains and growth condition.

E. amylovora Ea321 is a wild-type strain that infects pear and apple (Beer et al. 1991). *Escherichia coli* DH5α was routinely used for cloning of cosmids and plasmids. pCPP1001 (Wei and Beer 1995), pCPP1036 (Kim et al. 1997), pCPP1078 (Wei and Beer 1995), and pCPP1178 are subclones of pCPP430 (Beer et al. 1991), and contain ORFs in the same direction as the T7Φ10 promoter from the vector pBluescript KS+. Strains of *E. amylovora* Ea321 and *Escherichia coli* were grown in LB (Sambrook et al. 1989) with vigorous shaking at 28 and 37°C, respectively. Inducing medium (IM) was used for inducing *hrp* gene expression as described previously (Wei et al. 1992). The antibiotics used to maintain selection were ampicillin at 100 μg/ml, kanamycin (Km) at 50 μg/ml, spectinomycin (Sp) at 50 μg/ml, tetracycline (Tc) at 20 μg/ml, and carbenicillin (Cb) at 300 μg/ml.

Recombinant DNA techniques and sequence analysis.

Unless otherwise specified, basic molecular biology techniques were as described (Sambrook et al. 1989). Electroporation of plasmid DNA into *E. amylovora* 321 and its derivatives was done as described by Bauer and Beer (1991) with the Gene Pulser apparatus (Bio-Rad, Richmond, CA, U.S.A.).

Deletion clones, generated from the *ClaI-BglII* insert in pCPP1178 with the Erase-A-Base kit (Promega, Madison, WI, U.S.A.), were sequenced by the dideoxy chain termination procedure with the Sequenase sequencing kit (U.S. Biochemical, Cleveland, OH, U.S.A.). Also, sequencing of the region between *hrpA* and *hrpJ* in pCPP430, pCPP1001, pCPP1036, and pCPP1178 was performed on an ABI 373A automated DNA sequencer (Perkin-Elmer, Norwalk, CT, U.S.A.) at the

Cornell University Biotechnology Program DNA Sequencing Facility with oligonucleotide primers synthesized at the same facility.

DNA and deduced amino acid sequences were analyzed with programs in the GCG software package, version 7.3 (Genetics Computer Group, Madison, WI, U.S.A.) and DNASTAR (DNASTAR, Madison, WI, U.S.A.). Potential genes were identified with GeneMark.hmm (Lukashin and Borodovsky 1998; available on-line from the GeneMark web site). Homology searches were done with BLAST algorithms (Altschul et al. 1997; available on-line from the NCBI web site). Conserved patterns in proteins were found with ScanProsite (Appel et al. 1994; available on-line). Finally, prediction of potential σ^{70} promoters were made with the Promoter Prediction by Neural Network method (Reese and Eeckman 1995; available on-line).

Expression of *hrpX* and *hrpY* in *Escherichia coli*.

A gene expression system mediated by a T7 RNA polymerase/promoter (Tabor and Richardson 1985) was used. pCPP1178, which contains *hrpX* and *hrpY* ORFs driven by the T7Φ10 promoter from the vector, was introduced into *Escherichia coli* DH5α(pGP1-2). Cells were incubated at 42°C to induce the expression of the T7 RNA polymerase gene, and newly synthesized proteins were radiolabeled with ^{35}S-Met as described (Tabor and Richardson 1985). Resulting samples were resuspended in a sample buffer and heated to 95°C for 3 min before being electrophoresed in a 12% polyacrylamide gel.

Construction of marker-exchange mutants.

Chromosomal mutants were constructed by marker-exchange mutagenesis as described previously (Wei et al. 1992). A Tn*10*-miniKm insertion or a Tn*phoA* insertion, mapped at the *hrpXY* or *hrpL* locus in *Escherichia coli* DH5(pCCPP430) or *Escherichia coli* DH5α(pCPP1178), was introduced into *E. amylovora* Ea321 by triparental mating with the helper strain, *Escherichia coli* HB101(pRK600) (kindly provided by E. R. Signer; Department of Biology, Massachusetts Institute of Technology, Cambridge). The transconjugants were selected on Luria plates containing Km and Sp, and then transferred to a low-phosphate minimal medium (Bauer 1990) to select for Km^r Sp^s marker-exchanged mutants. The second mutations were generated by introducing individual *hrp*::Tn*5-gusA1* fusions into Tn*10*-miniKm or Tn*phoA* mutants of Ea321. Since the transposon Tn*5-gusA1* has two selection marker, Km and Tc, the second mutation was selected based on Km^r Tc^r Sp^s phenotype. All the mutants were tested for the HR-eliciting ability and pathogenicity. Tn*phoA* insertions P74 and P86 in pCPP1036, which were mapped to *orfU1*, were introduced to the Ea321 genome by electroporation and subsequent incubation in a low-phosphate medium with Km. Integration of the Tn*phoA* fusion into the chromosome was confirmed by antibiotic resistance (Km^r Cb^s) and Southern hybridization with the transposon DNA as a probe.

Assay of GUS activity.

Overnight cultures in LB were transferred to fresh LB, and incubated further. For incubation in IM, log-phase cultures in LB were centrifuged, and cells were washed with IM, before they are resuspended in IM to $OD_{620} \approx 0.5$. The cultures in IM were incubated for an additional 5 to 6 h at 24°C before assay of GUS activity. GUS activity was monitored fluorimetrically as described by Jefferson et al. (1987). Forty-five microliters of the log-phase culture in LB or the induced culture from IM was mixed with an equal volume of 2× assay buffer. After incubation at 37°C for 10 h, GUS activity was measured as described previously (Wei et al. 1992). The background fluorescence of Ea321-G77 (*hrcV*::Tn*5-gusA1*) (Wei et al. 1992), which has a *gusA1* insertion in the opposite direction of *hrcV* transcription, was subtracted from the readings of *hrp*::*gusA1* fusion strains. The corrected fluorescence readings were converted to picounits of GUS activity per CFU. The GUS activity of *hrp*::Tn*5-gusA1* fusions also were determined in tobacco leaf tissues as described previously (Wei et al. 1992).

Plant assays.

Bacteria were grown in LB and harvested at mid-exponential phase. Cells were resuspended in 5 mM potassium phosphate buffer, pH 6.5, harvested again, resuspended in the potassium phosphate buffer to approximately 2×10^8 CFU per ml, unless otherwise specified, and used for HR and pathogenicity assays. Tobacco plants (*Nicotiana tabacum* L. 'Xanthi') were grown in greenhouse soil mix to a height of 0.9 to 1 m. Bacterial suspensions were infiltrated into each leaf panel of tobacco leaves with needleless hypodermic syringes. The development of the HR was scored after incubation at room temperature for 18 to 36 h. Pathogenicity tests on immature pear fruits were carried out as previously described (Bauer and Beer 1991; Steinberger and Beer 1988).

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NOTE ADDED IN PROOF

A recent BLAST survey of finished and unfinished microbial genomes (available on-line from the NCBI web site) suggests the presence in *Pseudomonas aeruginosa* PAO1 of a two-component system that is highly similar to the HrpX/HrpY system (31% identity over 474 amino acids for HrpX and 48% identity over 208 amino acids for HrpY). A related set of proteins exist in the *Pseudomonas putida* KT2440 genome.

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Bacterial home goal by harpins

Ulla Bonas

Host–pathogen interactions are dynamic and multifactorial; whether a microorganism succeeds or fails in colonizing a potential host depends on factors from both organisms. A successful pathogen has to overcome the defenses of the host. In bacteria that are pathogenic for animals or for plants, particularly Gram-negative organisms, a large number of genes are essential to infect host tissue and establish disease. Expression of these genes is generally controlled by environmental conditions such as temperature, pH, salt concentration and nutrient availability[1,2].

Pathogenicity, hypersensitive reaction and elicitors

In the Gram-negative plant pathogens *Erwinia*, *Pseudomonas* and *Xanthomonas*, genes organized in clusters of 25–40 kb are fundamentally involved in any obvious interaction with a plant (for a review see Ref. 3). These genes have been designated *hrp* (hypersensitive reaction and pathogenicity) because they are essential not only for pathogenicity towards a susceptible host plant, but also for interaction with resistant host varieties and with plants that are not a host for that pathogen. In plants, the hypersensitive reaction (HR) (Ref. 4) is a rapid defense reaction involving localized plant cell death and production of substances such as phenolics and phytoalexins at the site of infection. The HR prevents pathogen spread and thus halts disease development.

In the wild, plants are resistant to the majority of pathogens. The HR, therefore, is an important defense mechanism against all kinds of possible disease agents (bacteria, fungi, nematodes and viruses). It is not only important to interactions of pathogens with nonhost plants, but also to interactions between plants that carry resistance genes and microorganisms that are pathogens for that species.

Although the genes involved in plant defense[5,6] are becoming better understood, very little is known about the nature of the initial signals and their perception. Induction of the HR in a bacterium–plant interaction requires functional *hrp* genes and appears to be mediated by signal molecules or 'elicitors'. Recent DNA sequence analyses indicate that several putative Hrp proteins from different species are related and may be involved in a secretion system reminiscent of secretion of Yops (*Yersinia* outer proteins) in *Yersinia*[7–11]. So far, only one specific elicitor of the HR in a bacterium–plant interaction has been described. The *avrD* gene from *Pseudomonas syringae* pv. *tomato* mediates production of a low-molecular-mass compound that specifically induces the HR only in the soybean plant (a nonhost) when it carries the corresponding *Rpg4* resistance gene[5,12].

Harpins

Recently, two bacterial HR-inducing proteins, called 'harpins', were identified in *Erwinia amylovora*[13] and *P. syringae* pv. *syringae*[14]. Although the harpins differ in primary sequence, they have several features in common: they are glycine rich and heat stable, and they both induce an HR in tobacco, a nonhost plant for these bacteria. The genes encoding harpins are localized within the *hrp* clusters and obviously have a dual role in that they are also required for pathogenicity towards the normal host plant. Both *hrp* clusters allow nonpathogenic bacteria, such as *Escherichia coli*, to induce an HR in tobacco after recombinant expression, suggesting that the genes for the tobacco HR elicitors are present within the clusters[15,16].

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The first harpin to be identified, harpin$_{Ea}$, is a cell-envelope-associated protein encoded by the *hrpN* gene of *Er. amylovora*, a pathogen of pear and apple[13]. Recently, He and co-workers[14] have used an elegant approach to identify harpin$_{Pss}$, which is encoded by the *hrpZ* gene in the bean pathogen *P. s.* pv. *syringae*. Lysates of an expression library in *E. coli*, made using the cloned *P. s.* pv. *syringae hrp* cluster, were directly screened for HR-inducing activity on tobacco leaves. Two proteins were identified, one of which was an amino-terminal deletion of harpin$_{Pss}$ with even higher activity than the full-size protein; whether processing occurs during natural infection is not clear. Interestingly, the carboxyl terminus contains two short, direct repeats that are essential for elicitor activity. The activity is in the same range as that of the *Erwinia* harpin$_{Ea}$; however, to elicit an HR in other plants requires higher levels of the elicitor. He *et al.* show convincingly that the secretion of harpin$_{Pss}$ by *P. s.* pv. *syringae* depends on a product called HrpH that is closely related to proteins in other plant pathogens, and also in animal pathogens such as *Yersinia* and *Shigella*, where they are essential for protein secretion[9,10,14].

These exciting findings help verify the model that Hrp proteins are involved in the transport of elicitors and virulence factors[7]. Not surprisingly, the results presented by He and co-workers[14] also stimulate many questions. It needs to be shown that harpin$_{Pss}$ is actually secreted when the bacterium interacts with tobacco tissue (the *hrp* genes were induced *in vitro*). The concentration needed for HR induction (more than 600 nM) is much higher than one would expect for specific signal molecules. Are harpins toxins? Most importantly, what is their function in pathogenicity, and why do they

not elicit an HR in the host plant? Are harpins the only elicitors of nonhost HR in tobacco and possibly in other plants? Is the same mechanism used in tobacco to recognize both the *Erwinia* and the *P. s.* pv. *syringae* harpins? Is host resistance different in mechanism from nonhost resistance? Answers to this fascinating puzzle require the identification of more HR elicitors and their putative plant receptors.

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Initiation and spread of α-herpesvirus infections

Thomas C. Mettenleiter

Herpesviruses are large animal viruses with a DNA genome varying from approximately 120 to 250 kb. Based on their biological properties, the Herpesviridae have been divided into three subfamilies, the α-, β- and γ-herpesvirinae, prototypes of which are the human pathogens herpes simplex virus (HSV), cytomegalovirus (HCMV) and Epstein–Barr virus (EBV), respectively. As enveloped viruses, they depend on two consecutive processes for infectious entry into target cells: (1) attachment of free virions to cells and (2) penetration, that is, fusion of virion envelope and cellular cytoplasmic membrane leading to release of the nucleocapsid into the cell. Virion envelope glycoproteins play important roles in both these processes (see Refs 1,2 for recent reviews).

After infection of primary target cells, virus spread can occur by several different mechanisms. Infected cells may release infectious virions that reinitiate infection from outside. In addition, direct viral cell-to-cell spread from primary infected cells to adjacent noninfected cells may occur. In the host, virus may be disseminated by circulating infected cells that adhere to noninfected tissues and transmit infectivity directly. Recent results on HSV and pseudorabies virus (PrV) shed more light on these processes in α-herpesviruses. PrV causes Aujeszky's disease in swine, which is characterized by nervous and respiratory symptoms, and reproductive failure. Unlike HSV, PrV is not pathogenic for humans. However, the two viruses have several features in common, including a broad host range *in vitro*, and several species besides the natural host can be infected experimentally. In addition, all of the known PrV glycoproteins are related to homologous glycoproteins in HSV (Ref. 1)*.

Attachment

Binding of free infectious virus to target cells involves interactions between virion envelope glycoproteins and cellular virus receptors. Herpes virions contain a large number of different virus-encoded envelope glycoproteins that might participate in attachment. A well-known example of a cellular herpesvirus receptor is the B-cell membrane protein CR2 (CD21), which binds EBV (Ref. 3). Recent studies have demonstrated that several α- (reviewed in Ref. 1), β- and γ-herpesviruses[4,5] bind to their target cells by interaction of virion components with cell-surface glycosaminoglycans, principally heparan sulfate (HS)[6].

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*At the 18th International Herpesvirus Workshop, a common nomenclature for α-herpesvirus glycoproteins was agreed on, based on designations of HSV glycoproteins. This nomenclature is used here.

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(54) Title: HYPERSENSITIVE RESPONSE ELICITING DOMAINS AND USE THEREOF

(57) Abstract: The present invention is directed to the structure of an isolated protein or polypeptide which elicits a hypersensitive response in plants as well as an isolated nucleic acid molecule which encodes the hypersensitive response eliciting protein or polypeptide. This protein or polypeptide has an acid portion linked to an alpha helix or a pair of spaced apart domains comprising an acidic portion linked to an alpha-helix. This isolated protein or polypeptide and the isolated nucleic acid molecule can be used to impart disease resistance to plants, to enhance plant growth, to control insects, and/or to impart stress resistance to plants. This can be achieved by applying the hypersensitive response elicitor protein or polypeptide in a non-infectious form to plants or plant seeds under conditions effective to impart disease resistance, to enhance plant growth, to control insects, and/or to impart stress resistance to plants or plants grown from the plant seeds. Alternatively, transgenic plants or plant seeds transformed with a nucleic acid molecule encoding a hypersensitive response elicitor protein or polypeptide can be provided and the transgenic plants or plants resulting from the transgenic plant seeds are grown under conditions effective to impart disease resistance, to enhance plant growth, to control insects, and/or to impart stress resistance to plants or plants grown from the plant seeds.

HYPERSENSITIVE RESPONSE ELICITING DOMAINS AND USE THEREOF

This application claims benefit of U.S. Provisional Patent Application Serial No. 60/212,211, filed on June 16, 2000.

FIELD OF THE INVENTION

The present invention relates to hypersensitive response elicitors and their structure.

BACKGROUND OF THE INVENTION

Interactions between bacterial pathogens and their plant hosts generally fall into two categories: (1) compatible (pathogen-host), leading to intercellular bacterial growth, symptom development, and disease development in the host plant; and (2) incompatible (pathogen-nonhost), resulting in the hypersensitive response, a particular type of incompatible interaction occurring, without progressive disease symptoms. During compatible interactions on host plants, bacterial populations increase dramatically and progressive symptoms occur. During incompatible interactions, bacterial populations do not increase, and progressive symptoms do not occur.

The hypersensitive response is a rapid, localized necrosis that is associated with the active defense of plants against many pathogens (Kiraly, Z., "Defenses Triggered by the Invader: Hypersensitivity," pages 201-224 in: Plant Disease: An Advanced Treatise, Vol. 5, J.G. Horsfall and E.B. Cowling, ed. Academic Press New York (1980); Klement, Z., "Hypersensitivity," pages 149-177 in: Phytopathogenic Prokaryotes, Vol. 2, M.S. Mount and G.H. Lacy, ed. Academic Press, New York (1982)). The hypersensitive response elicited by bacteria is readily observed as a tissue collapse if high concentrations ($\geq 10^7$ cells/ml) of a limited host-range pathogen like *Pseudomonas syringae* or *Erwinia amylovora* are infiltrated into the leaves of nonhost plants (necrosis occurs only in isolated plant cells at lower levels of inoculum) (Klement, Z., "Rapid Detection of Pathogenicity of Phytopathogenic Pseudomonads," Nature 199:299-300; Klement, et al.,

"Hypersensitive Reaction Induced by Phytopathogenic Bacteria in the Tobacco Leaf," Phytopathology 54:474-477 (1963); Turner, et al., "The Quantitative Relation Between Plant and Bacterial Cells Involved in the Hypersensitive Reaction," Phytopathology 64:885-890 (1974); Klement, Z., "Hypersensitivity," pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York (1982)). The capacities to elicit the hypersensitive response in a nonhost and be pathogenic in a host appear linked. As noted by Klement, Z., "Hypersensitivity," pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York, these pathogens also cause physiologically similar, albeit delayed, necroses in their interactions with compatible hosts. Furthermore, the ability to produce the hypersensitive response or pathogenesis is dependent on a common set of genes, denoted *hrp* (Lindgren, P.B., et al., "Gene Cluster of *Pseudomonas syringae* pv. 'phaseolicola' Controls Pathogenicity of Bean Plants and Hypersensitivity on Nonhost Plants," J. Bacteriol. 168:512-22 (1986); Willis, D.K., et al., "*hrp* Genes of Phytopathogenic Bacteria," Mol. Plant-Microbe Interact. 4:132-138 (1991)). Consequently, the hypersensitive response may hold clues to both the nature of plant defense and the basis for bacterial pathogenicity.

The *hrp* genes are widespread in gram-negative plant pathogens, where they are clustered, conserved, and in some cases interchangeable (Willis, D.K., et al., "*hrp* Genes of Phytopathogenic Bacteria," Mol. Plant-Microbe Interact. 4:132-138 (1991); Bonas, U., "*hrp* Genes of Phytopathogenic Bacteria," pages 79-98 in: Current Topics in Microbiology and Immunology: Bacterial Pathogenesis of Plants and Animals - Molecular and Cellular Mechanisms, J.L. Dangl, ed. Springer-Verlag, Berlin (1994)). Several *hrp* genes encode components of a protein secretion pathway similar to one used by *Yersinia, Shigella*, and *Salmonella* spp. to secrete proteins essential in animal diseases (Van Gijsegem, et al., "Evolutionary Conservation of Pathogenicity Determinants Among Plant and Animal Pathogenic Bacteria," Trends Microbiol. 1:175-180 (1993)). In *E. amylovora, P. syringae*, and *P. solanacearum*, hrp genes have been shown to control the production and secretion of glycine-rich, protein elicitors of the hypersensitive response (He, S.Y., et al. "Pseudomonas Syringae pv. Syringae HarpinPss: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266 (1993), Wei, Z.-H.,

et al., "HrpI of *Erwinia amylovora* Functions in Secretion of Harpin and is a Member of a New Protein Family," J. Bacteriol. 175:7958-7967 (1993); Arlat, M. et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum,*" EMBO J. 13:543-553 (1994)).

The first of these proteins was discovered in *E. amylovora* Ea321, a bacterium that causes fire blight of rosaceous plants, and was designated harpin (Wei, Z.-M., et al, "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992)). Mutations in the encoding *hrpN* gene revealed that harpin is required for *E. amylovora* to elicit a hypersensitive response in nonhost tobacco leaves and incite disease symptoms in highly susceptible pear fruit. The *P. solanacearum* GMI1000 PopA1 protein has similar physical properties and also elicits the hypersensitive response in leaves of tobacco, which is not a host of that strain (Arlat, et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum,*" EMBO J. 13:543-53 (1994)). However, *P. solanacearum popA* mutants still elicit the hypersensitive response in tobacco and incite disease in tomato. Thus, the role of these glycine-rich hypersensitive response elicitors can vary widely among gram-negative plant pathogens.

Other plant pathogenic hypersensitive response elicitors have been isolated, cloned, and sequenced. These include: *Erwinia chrysanthemi* (Bauer, et. al., "*Erwinia chrysanthemi* $Harpin_{Ech}$: Soft-Rot Pathogenesis," MPMI 8(4): 484-91 (1995)); *Erwinia carotovora* (Cui, et. al., "The RsmA$^-$ Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrp*N_{Ecc} and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI 9(7): 565-73 (1966)); *Erwinia stewartii* (Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microb. Inter. July 14-19, 1996 and Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," Ann. Mtg. Am. Phytopath. Soc. July 27-31, 1996); and *Pseudomonas syringae* pv. *syringae* (WO 94/26782 to Cornell Research Foundation, Inc.).

The present invention is a further advance in the effort to identify and characterize hypersensitive response elicitor proteins.

SUMMARY OF THE INVENTION

One aspect of the present invention is directed to an isolated hypersensitive response elicitor protein comprising a pair of spaced apart domains, with each comprising an acid portion linked to an alpha-helix.

Another embodiment of the present invention relates to an isolated hypersensitive response elicitor protein comprising an acid portion linked to an alpha-helix.

Nucleic acid molecules encoding either of these proteins as well as vectors, host cells, transgenic plants, and transgenic plant seeds containing those nucleic acid molecules are also disclosed.

The protein of the present invention can be used to impart disease resistance to plants, to enhance plant growth, to control insects, and/or impart stress resistance. This involves applying the protein to plants or plant seeds under conditions effective to impart disease resistance, to enhance plant growth, to control insects, and/or impart stress resistance to plants or plants grown from the plant seeds.

As an alternative to applying the protein to plants or plant seeds in order to impart disease resistance, to enhance plant growth, to control insects on plants, and/or impart stress resistance, transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a nucleic acid molecule encoding the protein of the present invention and growing the plant under conditions effective to impart disease resistance, to enhance plant growth, to control insects, and/or to impart stress resistance to the plants or plants grown from the plant seeds. Alternatively, a transgenic plant seed transformed with the nucleic acid molecule encoding the protein of the present invention can be provided and planted in soil. A plant is then propagated under conditions effective to impart disease resistance, to enhance plant growth, to control insects, and/or to impart stress resistance to plants or plants grown from the plant seeds.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic drawing showing the construction of a universal expression cassette for a hypersensitive response domain.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to an isolated hypersensitive response elicitor protein comprising a pair of spaced apart domains, with each comprising an acid portion linked to an alpha-helix. The acidic portion is a polypeptide with 10 or more amino acids, is rich in acidic amino acids, and has a pI below 5.0. The acidic portion has a secondary structure in the form of a beta-sheet or a beta-turn. The secondary structure of this unit can also be in an unordered form.

The alpha-helix portion of the present invention is a polypeptide with 10 or more amino acids. Its secondary structure is in the form of a stable alpha-helix.

Another embodiment of the present invention relates to an isolated hypersensitive response elicitor protein comprising an acid portion linked to an alpha-helix.

Both of these proteins are capable of eliciting a hypersensitive response.

The alpha helix is a common structural motif of proteins in which a linear sequence of amino acid folds into a right-handed helix stabilized by internal hydrogen bonding between backbone atoms.

The acidic motif includes a certain combination of amino acids in which a linear sequence with a pI below 5.0 folds into a β sheet, coil, or thin structures but not an alpha helix of secondary structure.

The hypersensitive response elicitor polypeptides or proteins according to the present invention can be derived from hypersensitive response elicitor polypeptides or proteins of a wide variety of fungal and bacterial pathogens. Such polypeptides or proteins are able to elicit local necrosis in plant tissue contacted by the elicitor. Examples of suitable bacterial sources of polypeptide or protein elicitors

include *Erwinia*, *Pseudomonas*, and *Xanthamonas* species (e.g., the following bacteria: *Erwinia amylovora, Erwinia chrysanthemi, Erwinia stewartii, Erwinia carotovora, Pseudomonas syringae, Pseudomonas solancearum, Xanthomonas campestris*, and mixtures thereof). In addition to hypersensitive response elicitors from these Gram negative bacteria, it is possible to use elicitors from Gram positive bacteria. One example is *Clavibacter michiganensis* subsp. *sepedonicus*.

An example of a fungal source of a hypersensitive response elicitor protein or polypeptide is *Phytophthora*. Suitable species of *Phytophthora* include *Phytophthora parasitica, Phytophthora cryptogea, Phytophthora cinnamomi, Phytophthora capsici, Phytophthora megasperma*, and *Phytophthora citrophthora*.

The hypersensitive response elicitor polypeptide or protein from *Erwinia chrysanthemi* has an amino acid sequence corresponding to SEQ. ID. No. 1 as follows:

```
Met Gln Ile Thr Ile Lys Ala His Ile Gly Gly Asp Leu Gly Val Ser
1               5                  10                  15

Gly Leu Gly Ala Gln Gly Leu Lys Gly Leu Asn Ser Ala Ala Ser Ser
            20                  25                  30

Leu Gly Ser Ser Val Asp Lys Leu Ser Ser Thr Ile Asp Lys Leu Thr
        35                  40                  45

Ser Ala Leu Thr Ser Met Met Phe Gly Gly Ala Leu Ala Gln Gly Leu
    50                  55                  60

Gly Ala Ser Ser Lys Gly Leu Gly Met Ser Asn Gln Leu Gly Gln Ser
65                  70                  75                  80

Phe Gly Asn Gly Ala Gln Gly Ala Ser Asn Leu Leu Ser Val Pro Lys
                85                  90                  95

Ser Gly Gly Asp Ala Leu Ser Lys Met Phe Asp Lys Ala Leu Asp Asp
            100                 105                 110

Leu Leu Gly His Asp Thr Val Thr Lys Leu Thr Asn Gln Ser Asn Gln
        115                 120                 125

Leu Ala Asn Ser Met Leu Asn Ala Ser Gln Met Thr Gln Gly Asn Met
    130                 135                 140

Asn Ala Phe Gly Ser Gly Val Asn Asn Ala Leu Ser Ser Ile Leu Gly
145                 150                 155                 160

Asn Gly Leu Gly Gln Ser Met Ser Gly Phe Ser Gln Pro Ser Leu Gly
                165                 170                 175
```

```
Ala Gly Gly Leu Gln Gly Leu Ser Gly Ala Gly Ala Phe Asn Gln Leu
            180                 185                 190

Gly Asn Ala Ile Gly Met Gly Val Gly Gln Asn Ala Ala Leu Ser Ala
        195                 200                 205

Leu Ser Asn Val Ser Thr His Val Asp Gly Asn Asn Arg His Phe Val
    210                 215                 220

Asp Lys Glu Asp Arg Gly Met Ala Lys Glu Ile Gly Gln Phe Met Asp
225                 230                 235                 240

Gln Tyr Pro Glu Ile Phe Gly Lys Pro Glu Tyr Gln Lys Asp Gly Trp
                245                 250                 255

Ser Ser Pro Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser Lys
            260                 265                 270

Pro Asp Asp Asp Gly Met Thr Gly Ala Ser Met Asp Lys Phe Arg Gln
        275                 280                 285

Ala Met Gly Met Ile Lys Ser Ala Val Ala Gly Asp Thr Gly Asn Thr
    290                 295                 300

Asn Leu Asn Leu Arg Gly Ala Gly Gly Ala Ser Leu Gly Ile Asp Ala
305                 310                 315                 320

Ala Val Val Gly Asp Lys Ile Ala Asn Met Ser Leu Gly Lys Leu Ala
                325                 330                 335

Asn Ala
```

This hypersensitive response elicitor polypeptide or protein has a molecular weight of 34 kDa, is heat stable, has a glycine content of greater than 16%, and contains substantially no cysteine. The *Erwinia chrysanthemi* hypersensitive response elicitor polypeptide or protein is encoded by a DNA molecule having a nucleotide sequence corresponding to SEQ. ID. No. 2 as follows:

```
CGATTTTACC CGGGTGAACG TGCTATGACC GACAGCATCA CGGTATTCGA CACCGTTACG      60

GCGTTTATGG CCGCGATGAA CCGGCATCAG GCGGCGCGCT GGTCGCCGCA ATCCGGCGTC     120

GATCTGGTAT TTCAGTTTGG GGACACCGGG CGTGAACTCA TGATGCAGAT TCAGCCGGGG     180

CAGCAATATC CCGGCATGTT GCGCACGCTG CTCGCTCGTC GTTATCAGCA GGCGGCAGAG     240

TGCGATGGCT GCCATCTGTG CCTGAACGGC AGCGATGTAT TGATCCTCTG GTGGCCGCTG     300

CCGTCGGATC CCGGCAGTTA TCCGCAGGTG ATCGAACGTT TGTTTGAACT GGCGGGAATG     360

ACGTTGCCGT CGCTATCCAT AGCACCGACG GCGCGTCCGC AGACAGGGAA CGGACGCGCC     420

CGATCATTAA GATAAAGGCG GCTTTTTTTA TTGCAAAACG GTAACGGTGA GGAACCGTTT     480
```

CACCGTCGGC GTCACTCAGT AACAAGTATC CATCATGATG CCTACATCGG GATCGGCGTG 540

GGCATCCGTT GCAGATACTT TTGCGAACAC CTGACATGAA TGAGGAAACG AAATTATGCA 600

AATTACGATC AAAGCGCACA TCGGCGGTGA TTTGGGCGTC TCCGGTCTGG GGCTGGGTGC 660

TCAGGGACTG AAAGGACTGA ATTCCGCGGC TTCATCGCTG GGTTCCAGCG TGGATAAACT 720

GAGCAGCACC ATCGATAAGT TGACCTCCGC GCTGACTTCG ATGATGTTTG GCGGCGCGCT 780

GGCGCAGGGG CTGGGCGCCA GCTCGAAGGG GCTGGGGATG AGCAATCAAC TGGGCCAGTC 840

TTTCGGCAAT GGCGCGCAGG GTGCGAGCAA CCTGCTATCC GTACCGAAAT CCGGCGGCGA 900

TGCGTTGTCA AAAATGTTTG ATAAAGCGCT GGACGATCTG CTGGGTCATG ACACCGTGAC 960

CAAGCTGACT AACCAGAGCA ACCAACTGGC TAATTCAATG CTGAACGCCA GCCAGATGAC 1020

CCAGGGTAAT ATGAATGCGT TCGGCAGCGG TGTGAACAAC GCACTGTCGT CCATTCTCGG 1080

CAACGGTCTC GGCCAGTCGA TGAGTGGCTT CTCTCAGCCT TCTCTGGGGG CAGGCGGCTT 1140

GCAGGGCCTG AGCGGCGCGG GTGCATTCAA CCAGTTGGGT AATGCCATCG GCATGGGCGT 1200

GGGGCAGAAT GCTGCGCTGA GTGCGTTGAG TAACGTCAGC ACCCACGTAG ACGGTAACAA 1260

CCGCCACTTT GTAGATAAAG AAGATCGCGG CATGGCGAAA GAGATCGGCC AGTTTATGGA 1320

TCAGTATCCG GAAATATTCG GTAAACCGGA ATACCAGAAA GATGGCTGGA GTTCGCCGAA 1380

GACGGACGAC AAATCCTGGG CTAAAGCGCT GAGTAAACCG GATGATGACG GTATGACCGG 1440

CGCCAGCATG GACAAATTCC GTCAGGCGAT GGGTATGATC AAAAGCGCGG TGGCGGGTGA 1500

TACCGGCAAT ACCAACCTGA ACCTGCGTGG CGCGGGCGGT GCATCGCTGG GTATCGATGC 1560

GGCTGTCGTC GGCGATAAAA TAGCCAACAT GTCGCTGGGT AAGCTGGCCA ACGCCTGATA 1620

ATCTGTGCTG GCCTGATAAA GCGGAAACGA AAAAAGAGAC GGGGAAGCCT GTCTCTTTTC 1680

TTATTATGCG GTTTATGCGG TTACCTGGAC CGGTTAATCA TCGTCATCGA TCTGGTACAA 1740

ACGCACATTT TCCCGTTCAT TCGCGTCGTT ACGCGCCACA ATCGCGATGG CATCTTCCTC 1800

GTCGCTCAGA TTGCGCGGCT GATGGGGAAC GCCGGGTGGA ATATAGAGAA ACTCGCCGGC 1860

CAGATGGAGA CACGTCTGCG ATAAATCTGT GCCGTAACGT GTTTCTATCC GCCCCTTTAG 1920

CAGATAGATT GCGGTTTCGT AATCAACATG GTAATGCGGT TCCGCCTGTG CGCCGGCCGG 1980

GATCACCACA ATATTCATAG AAAGCTGTCT TGCACCTACC GTATCGCGGG AGATACCGAC 2040

AAAATAGGGC AGTTTTTGCG TGGTATCCGT GGGGTGTTCC GGCCTGACAA TCTTGAGTTG 2100

GTTCGTCATC ATCTTTCTCC ATCTGGGCGA CCTGATCGGT T 2141

The hypersensitive response elicitor from *Erwinia chrysanthemi* has 2 hypersensitive response eliciting domains. The first domain extends, within SEQ.

ID. No. 1, from amino acid 69 to amino acid 122, particularly from amino acid 85 to amino acid 116. The acidic unit in the first domain extends, within SEQ. ID. No. 1, from amino acid 69 to amino acid 102, particularly from amino acid 85 to amino acid 102. The alpha-helix in the first domain extends, within SEQ. ID. No. 1, from amino acid 102 to amino acid 122, particularly from amino acid 102 to amino acid 116. The second domain extends, within SEQ. ID. No. 1, from amino acid 251 to amino acid 299, particularly from amino acid 256 to amino acid 292. The acidic unit in the second domain extends, within SEQ. ID. No. 1, from amino acid 251 to amino acid 279, particularly from amino acid 261 to amino acid 279. The alpha-helix in the second domain extends, within SEQ. ID. No. 1, from amino acid 279 to amino acid 299, particularly from amino acid 279 to amino acid 292.

The hypersensitive response elicitor polypeptide or protein derived from *Erwinia amylovora* has an amino acid sequence corresponding to SEQ. ID. No. 3 as follows:

```
Met Ser Leu Asn Thr Ser Gly Leu Gly Ala Ser Thr Met Gln Ile Ser
1               5                  10                  15

Ile Gly Gly Ala Gly Gly Asn Asn Gly Leu Leu Gly Thr Ser Arg Gln
            20                  25                  30

Asn Ala Gly Leu Gly Gly Asn Ser Ala Leu Gly Leu Gly Gly Gly Asn
        35                  40                  45

Gln Asn Asp Thr Val Asn Gln Leu Ala Gly Leu Leu Thr Gly Met Met
    50                  55                  60

Met Met Met Ser Met Met Gly Gly Gly Gly Leu Met Gly Gly Gly Leu
65                  70                  75                  80

Gly Gly Gly Leu Gly Asn Gly Leu Gly Gly Ser Gly Gly Leu Gly Glu
                85                  90                  95

Gly Leu Ser Asn Ala Leu Asn Asp Met Leu Gly Gly Ser Leu Asn Thr
            100                 105                 110

Leu Gly Ser Lys Gly Gly Asn Asn Thr Thr Ser Thr Thr Asn Ser Pro
        115                 120                 125

Leu Asp Gln Ala Leu Gly Ile Asn Ser Thr Ser Gln Asn Asp Asp Ser
    130                 135                 140

Thr Ser Gly Thr Asp Ser Thr Ser Asp Ser Ser Asp Pro Met Gln Gln
145                 150                 155                 160
```

```
Leu Leu Lys Met Phe Ser Glu Ile Met Gln Ser Leu Phe Gly Asp Gly
                165                 170                 175

Gln Asp Gly Thr Gln Gly Ser Ser Ser Gly Gly Lys Gln Pro Thr Glu
            180                 185                 190

Gly Glu Gln Asn Ala Tyr Lys Lys Gly Val Thr Asp Ala Leu Ser Gly
        195                 200                 205

Leu Met Gly Asn Gly Leu Ser Gln Leu Leu Gly Asn Gly Gly Leu Gly
    210                 215                 220

Gly Gly Gln Gly Gly Asn Ala Gly Thr Gly Leu Asp Gly Ser Ser Leu
225                 230                 235                 240

Gly Gly Lys Gly Leu Gln Asn Leu Ser Gly Pro Val Asp Tyr Gln Gln
                245                 250                 255

Leu Gly Asn Ala Val Gly Thr Gly Ile Gly Met Lys Ala Gly Ile Gln
            260                 265                 270

Ala Leu Asn Asp Ile Gly Thr His Arg His Ser Ser Thr Arg Ser Phe
        275                 280                 285

Val Asn Lys Gly Asp Arg Ala Met Ala Lys Glu Ile Gly Gln Phe Met
    290                 295                 300

Asp Gln Tyr Pro Glu Val Phe Gly Lys Pro Gln Tyr Gln Lys Gly Pro
305                 310                 315                 320

Gly Gln Glu Val Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser
                325                 330                 335

Lys Pro Asp Asp Asp Gly Met Thr Pro Ala Ser Met Glu Gln Phe Asn
            340                 345                 350

Lys Ala Lys Gly Met Ile Lys Arg Pro Met Ala Gly Asp Thr Gly Asn
        355                 360                 365

Gly Asn Leu Gln Ala Arg Gly Ala Gly Gly Ser Ser Leu Gly Ile Asp
    370                 375                 380

Ala Met Met Ala Gly Asp Ala Ile Asn Asn Met Ala Leu Gly Lys Leu
385                 390                 395                 400

Gly Ala Ala
```

This hypersensitive response elicitor polypeptide or protein has a molecular weight of about 39 kDa, has a pI of approximately 4.3, and is heat stable at 100°C for at least 10 minutes. This hypersensitive response elicitor polypeptide or protein has substantially no cysteine. The hypersensitive response elicitor polypeptide or protein derived from *Erwinia amylovora* is more fully described in Wei, Z.-M., R. J. Laby, C. H. Zumoff,

D. W. Bauer, S.-Y. He, A. Collmer, and S. V. Beer, "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992), which is hereby incorporated by reference. The DNA molecule encoding this polypeptide or protein has a nucleotide sequence corresponding to SEQ. ID. No. 4 as follows:

```
AAGCTTCGGC ATGGCACGTT TGACCGTTGG GTCGGCAGGG TACGTTTGAA TTATTCATAA      60
GAGGAATACG TTATGAGTCT GAATACAAGT GGGCTGGGAG CGTCAACGAT GCAAATTTCT     120
ATCGGCGGTG CGGGCGGAAA TAACGGGTTG CTGGGTACCA GTCGCCAGAA TGCTGGGTTG     180
GGTGGCAATT CTGCACTGGG GCTGGGCGGC GGTAATCAAA ATGATACCGT CAATCAGCTG     240
GCTGGCTTAC TCACCGGCAT GATGATGATG ATGAGCATGA TGGGCGGTGG TGGGCTGATG     300
GGCGGTGGCT TAGGCGGTGG CTTAGGTAAT GGCTTGGGTG GCTCAGGTGG CCTGGGCGAA     360
GGACTGTCGA ACGCGCTGAA CGATATGTTA GGCGGTTCGC TGAACACGCT GGGCTCGAAA     420
GGCGGCAACA ATACCACTTC AACAACAAAT TCCCCGCTGG ACCAGGCGCT GGGTATTAAC     480
TCAACGTCCC AAAACGACGA TTCCACCTCC GGCACAGATT CCACCTCAGA CTCCAGCGAC     540
CCGATGCAGC AGCTGCTGAA GATGTTCAGC GAGATAATGC AAAGCCTGTT TGGTGATGGG     600
CAAGATGGCA CCCAGGGCAG TTCCTCTGGG GGCAAGCAGC CGACCGAAGG CGAGCAGAAC     660
GCCTATAAAA AAGGAGTCAC TGATGCGCTG TCGGGCCTGA TGGGTAATGG TCTGAGCCAG     720
CTCCTTGGCA ACGGGGGACT GGGAGGTGGT CAGGGCGGTA ATGCTGGCAC GGGTCTTGAC     780
GGTTCGTCGC TGGGCGGCAA AGGGCTGCAA AACCTGAGCG GGCCGGTGGA CTACCAGCAG     840
TTAGGTAACG CCGTGGGTAC CGGTATCGGT ATGAAAGCGG GCATTCAGGC GCTGAATGAT     900
ATCGGTACGC ACAGGCACAG TTCAACCCGT TCTTTCGTCA ATAAAGGCGA TCGGGCGATG     960
GCGAAGGAAA TCGGTCAGTT CATGGACCAG TATCCTGAGG TGTTTGGCAA GCCGCAGTAC    1020
CAGAAAGGCC CGGGTCAGGA GGTGAAAACC GATGACAAAT CATGGGCAAA AGCACTGAGC    1080
AAGCCAGATG ACGACGGAAT GACACCAGCC AGTATGGAGC AGTTCAACAA AGCCAAGGGC    1140
ATGATCAAAA GGCCCATGGC GGGTGATACC GGCAACGGCA ACCTGCAGGC ACGCGGTGCC    1200
GGTGGTTCTT CGCTGGGTAT TGATGCCATG ATGGCCGGTG ATGCCATTAA CAATATGGCA    1260
CTTGGCAAGC TGGGCGCGGC TTAAGCTT                                       1288
```

The hypersensitive response elicitor from *Erwinia amylovora* has 2 hypersensitive response eliciting domains. The first domain extends, within SEQ. ID. No. 3, from amino acid 32 to amino acid 74, particularly from amino acid 45 to amino

acid 68. The acidic unit in the first domain extends, within SEQ. ID. No. 3, from amino acid 32 to amino acid 57, particularly from amino acid 45 to amino acid 57. The alpha-helix in the first domain extends, within SEQ. ID. No. 3, from amino acid 57 to amino acid 74, particularly from amino acid 57 to amino acid 68. The second domain extends, within SEQ. ID. No. 3, from amino acid 130 to amino acid 180, particularly from amino acid 145 to amino acid 170. The acidic unit in the second domain extends, within SEQ. ID. No. 3, from amino acid 130 to amino acid 157, particularly from amino acid 145 to amino acid 157. The alpha-helix in the second domain extends, within SEQ. ID. No. 3, from amino acid 157 to amino acid 180, particularly from amino acid 157 to amino acid 170.

Another potentially suitable hypersensitive response elicitor from *Erwinia amylovora* is disclosed in U.S. Patent Application Serial No. 09/120,927, which is hereby incorporated by reference. The protein is encoded by a DNA molecule having a nucleic acid sequence of SEQ. ID. No. 5 as follows:

```
ATGTCAATTC TTACGCTTAA CAACAATACC TCGTCCTCGC CGGGTCTGTT CCAGTCCGGG      60
GGGGACAACG GGCTTGGTGG TCATAATGCA AATTCTGCGT TGGGGCAACA ACCCATCGAT     120
CGGCAAACCA TTGAGCAAAT GGCTCAATTA TTGGCGGAAC TGTTAAAGTC ACTGCTATCG     180
CCACAATCAG GTAATGCGGC AACCGGAGCC GGTGGCAATG ACCAGACTAC AGGAGTTGGT     240
AACGCTGGCG GCCTGAACGG ACGAAAAGGC ACAGCAGGAA CCACTCCGCA GTCTGACAGT     300
CAGAACATGC TGAGTGAGAT GGGCAACAAC GGGCTGGATC AGGCCATCAC GCCCGATGGC     360
CAGGGCGGCG GGCAGATCGG CGATAATCCT TTACTGAAAG CCATGCTGAA GCTTATTGCA     420
CGCATGATGG ACGGCCAAAG CGATCAGTTT GGCCAACCTG GTACGGGCAA CAACAGTGCC     480
TCTTCCGGTA CTTCTTCATC TGGCGGTTCC CCTTTTAACG ATCTATCAGG GGGGAAGGCC     540
CCTTCCGGCA ACTCCCCTTC CGGCAACTAC TCTCCCGTCA GTACCTTCTC ACCCCCATCC     600
ACGCCAACGT CCCCTACCTC ACCGCTTGAT TTCCCTTCTT CTCCCACCAA AGCAGCCGGG     660
GGCAGCACGC CGGTAACCGA TCATCCTGAC CCTGTTGGTA GCGCGGGCAT CGGGGCCGGA     720
AATTCGGTGG CCTTCACCAG CGCCGGCGCT AATCAGACGG TGCTGCATGA CACCATTACC     780
GTGAAAGCGG GTCAGGTGTT TGATGGCAAA GGACAAACCT TCACCGCCGG TTCAGAATTA     840
GGCGATGGCG GCCAGTCTGA AAACCAGAAA CCGCTGTTTA TACTGGAAGA CGGTGCCAGC     900
CTGAAAAACG TCACCATGGG CGACGACGGG GCGGATGGTA TTCATCTTTA CGGTGATGCC     960
AAAATAGACA ATCTGCACGT CACCAACGTG GGTGAGGACG CGATTACCGT TAAGCCAAAC    1020
AGCGCGGGCA AAAAATCCCA CGTTGAAATC ACTAACAGTT CCTTCGAGCA CGCCTCTGAC    1080
```

```
AAGATCCTGC AGCTGAATGC CGATACTAAC CTGAGCGTTG ACAACGTGAA GGCCAAAGAC    1140

TTTGGTACTT TTGTACGCAC TAACGGCGGT CAACAGGGTA ACTGGGATCT GAATCTGAGC    1200

CATATCAGCG CAGAAGACGG TAAGTTCTCG TTCGTTAAAA GCGATAGCGA GGGGCTAAAC    1260

GTCAATACCA GTGATATCTC ACTGGGTGAT GTTGAAAACC ACTACAAAGT GCCGATGTCC    1320

GCCAACCTGA AGGTGGCTGA ATGA                                           1344
```

See GenBank Accession No. U94513. The isolated DNA molecule of the present invention encodes a hypersensitive response elicitor protein or polypeptide having an amino acid sequence of SEQ. ID. No. 6 as follows:

```
Met Ser Ile Leu Thr Leu Asn Asn Asn Thr Ser Ser Ser Pro Gly Leu
1               5                  10                  15

Phe Gln Ser Gly Gly Asp Asn Gly Leu Gly Gly His Asn Ala Asn Ser
            20                  25                  30

Ala Leu Gly Gln Gln Pro Ile Asp Arg Gln Thr Ile Glu Gln Met Ala
        35                  40                  45

Gln Leu Leu Ala Glu Leu Leu Lys Ser Leu Leu Ser Pro Gln Ser Gly
    50                  55                  60

Asn Ala Ala Thr Gly Ala Gly Gly Asn Asp Gln Thr Thr Gly Val Gly
65                  70                  75                  80

Asn Ala Gly Gly Leu Asn Gly Arg Lys Gly Thr Ala Gly Thr Thr Pro
                85                  90                  95

Gln Ser Asp Ser Gln Asn Met Leu Ser Glu Met Gly Asn Asn Gly Leu
            100                 105                 110

Asp Gln Ala Ile Thr Pro Asp Gly Gln Gly Gly Gly Gln Ile Gly Asp
        115                 120                 125

Asn Pro Leu Leu Lys Ala Met Leu Lys Leu Ile Ala Arg Met Met Asp
    130                 135                 140

Gly Gln Ser Asp Gln Phe Gly Gln Pro Gly Thr Gly Asn Asn Ser Ala
145                 150                 155                 160

Ser Ser Gly Thr Ser Ser Ser Gly Gly Ser Pro Phe Asn Asp Leu Ser
                165                 170                 175

Gly Gly Lys Ala Pro Ser Gly Asn Ser Pro Ser Gly Asn Tyr Ser Pro
            180                 185                 190

Val Ser Thr Phe Ser Pro Pro Ser Thr Pro Thr Ser Pro Thr Ser Pro
        195                 200                 205

Leu Asp Phe Pro Ser Ser Pro Thr Lys Ala Ala Gly Gly Ser Thr Pro
    210                 215                 220
```

```
Val Thr Asp His Pro Asp Pro Val Gly Ser Ala Gly Ile Gly Ala Gly
225                 230                 235                 240

Asn Ser Val Ala Phe Thr Ser Ala Gly Ala Asn Gln Thr Val Leu His
                245                 250                 255

Asp Thr Ile Thr Val Lys Ala Gly Gln Val Phe Asp Gly Lys Gly Gln
            260                 265                 270

Thr Phe Thr Ala Gly Ser Glu Leu Gly Asp Gly Gly Gln Ser Glu Asn
        275                 280                 285

Gln Lys Pro Leu Phe Ile Leu Glu Asp Gly Ala Ser Leu Lys Asn Val
    290                 295                 300

Thr Met Gly Asp Asp Gly Ala Asp Gly Ile His Leu Tyr Gly Asp Ala
305                 310                 315                 320

Lys Ile Asp Asn Leu His Val Thr Asn Val Gly Glu Asp Ala Ile Thr
                325                 330                 335

Val Lys Pro Asn Ser Ala Gly Lys Lys Ser His Val Glu Ile Thr Asn
            340                 345                 350

Ser Ser Phe Glu His Ala Ser Asp Lys Ile Leu Gln Leu Asn Ala Asp
        355                 360                 365

Thr Asn Leu Ser Val Asp Asn Val Lys Ala Lys Asp Phe Gly Thr Phe
    370                 375                 380

Val Arg Thr Asn Gly Gly Gln Gln Gly Asn Trp Asp Leu Asn Leu Ser
385                 390                 395                 400

His Ile Ser Ala Glu Asp Gly Lys Phe Ser Phe Val Lys Ser Asp Ser
                405                 410                 415

Glu Gly Leu Asn Val Asn Thr Ser Asp Ile Ser Leu Gly Asp Val Glu
            420                 425                 430

Asn His Tyr Lys Val Pro Met Ser Ala Asn Leu Lys Val Ala Glu
        435                 440                 445
```

This protein or polypeptide is acidic, rich in glycine and serine, and lacks cysteine. It is also heat stable, protease sensitive, and suppressed by inhibitors of plant metabolism. The protein or polypeptide of the present invention has a predicted molecular size of ca. 4.5 kDa.

This hypersensitive response elicitor from *Erwinia amylovora* has 2 hypersensitive response eliciting domains. The first domain extends, within SEQ. ID. No. 6, from amino acid 5 to amino acid 64, particularly from amino acid 31 to amino acid 57. The acidic unit in the first domain extends, within SEQ. ID. No. 6, from amino acid 5 to amino acid 45, particularly from amino acid 31 to amino acid 45. The

alpha-helix in the first domain extends, within SEQ. ID. No. 6, from amino acid 45 to amino acid 64, particularly from amino acid 45 to amino acid 64. The second domain extends, within SEQ. ID. No. 6, from amino acid 103 to amino acid 146, particularly from amino acid 116 to amino acid 140. The acidic unit in the second domain extends, within SEQ. ID. No. 6, from amino acid 103 to amino acid 131, particularly from amino acid 116 to amino acid 131. The alpha-helix in the second domain extends, within SEQ. ID. No. 6, from amino acid 131 to amino acid 146, particularly from amino acid 131 to amino acid 140.

Another potentially suitable hypersensitive response elicitor from *Erwinia amylovora* is disclosed in U.S. Patent Application Serial No. 09/120,663, which is hereby incorporated by reference. The protein is encoded by a DNA molecule having a nucleic acid sequence of SEQ. ID. No. 7 as follows:

```
ATGGAATTAA AATCACTGGG AACTGAACAC AAGGCGGCAG TACACACAGC GGCGCACAAC      60
CCTGTGGGGC ATGGTGTTGC CTTACAGCAG GGCAGCAGCA GCAGCAGCCC GCAAAATGCC     120
GCTGCATCAT TGGCGGCAGA AGGCAAAAAT CGTGGGAAAA TGCCGAGAAT TCACCAGCCA     180
TCTACTGCGG CTGATGGTAT CAGCGCTGCT CACCAGCAAA AGAAATCCTT CAGTCTCAGG     240
GGCTGTTTGG GGACGAAAAA ATTTTCCAGA TCGGCACCGC AGGGCCAGCC AGGTACCACC     300
CACAGCAAAG GGGCAACATT GCGCGATCTG CTGGCGCGGG ACGACGGCGA AACGCAGCAT     360
GAGGCGGCCG CGCCAGATGC GGCGCGTTTG ACCCGTTCGG GCGGCGTCAA ACGCCGCAAT     420
ATGGACGACA TGGCCGGGCG GCCAATGGTG AAAGGTGGCA GCGGCGAAGA TAAGGTACCA     480
ACGCAGCAAA AACGGCATCA GCTGAACAAT TTTGGCCAGA TGCGCCAAAC GATGTTGAGC     540
AAAATGGCTC ACCCGGCTTC AGCCAACGCC GGCGATCGCC TGCAGCATTC ACCGCCGCAC     600
ATCCCGGGTA GCCACCACGA AATCAAGGAA GAACCGGTTG GCTCCACCAG CAAGGCAACA     660
ACGGCCCACG CAGACAGAGT GGAAATCGCT CAGGAAGATG ACGACAGCGA ATTCCAGCAA     720
CTGCATCAAC AGCGGCTGGC GCGCGAACGG GAAAATCCAC CGCAGCCGCC CAAACTCGGC     780
GTTGCCACAC CGATTAGCGC CAGGTTTCAG CCCAAACTGA CTGCGGTTGC GGAAAGCGTC     840
CTTGAGGGGA CAGATACCAC GCAGTCACCC CTTAAGCCGC AATCAATGCT GAAAGGAAGT     900
GGAGCCGGGG TAACGCCGCT GGCGGTAACG CTGGATAAAG GCAAGTTGCA GCTGGCACCG     960
GATAATCCAC CCGCGCTCAA TACGTTGTTG AAGCAGACAT TGGGTAAAGA CACCCAGCAC    1020
TATCTGGCGC ACCATGCCAG CAGCGACGGT AGCCAGCATC TGCTGCTGGA CAACAAAGGC    1080
CACCTGTTTG ATATCAAAAG CACCGCCACC AGCTATAGCG TGCTGCACAA CAGCCACCCC    1140
GGTGAGATAA AGGGCAAGCT GGCGCAGGCG GGTACTGGCT CCGTCAGCGT AGACGGTAAA    1200
```

AGCGGCAAGA TCTCGCTGGG GAGCGGTACG CAAAGTCACA ACAAAACAAT GCTAAGCCAA 1260

CCGGGGGAAG CGCACCGTTC CTTATTAACC GGCATTTGGC AGCATCCTGC TGGCGCAGCG 1320

CGGCCGCAGG GCGAGTCAAT CCGCCTGCAT GACGACAAAA TTCATATCCT GCATCCGGAG 1380

CTGGGCGTAT GGCAATCTGC GGATAAAGAT ACCCACAGCC AGCTGTCTCG CCAGGCAGAC 1440

GGTAAGCTCT ATGCGCTGAA AGACAACCGT ACCCTGCAAA ACCTCTCCGA TAATAAATCC 1500

TCAGAAAAGC TGGTCGATAA AATCAAATCG TATTCCGTTG ATCAGCGGGG GCAGGTGGCG 1560

ATCCTGACGG ATACTCCCGG CCGCCATAAG ATGAGTATTA TGCCCTCGCT GGATGCTTCC 1620

CCGGAGAGCC ATATTTCCCT CAGCCTGCAT TTTGCCGATG CCCACCAGGG GTTATTGCAC 1680

GGGAAGTCGG AGCTTGAGGC ACAATCTGTC GCGATCAGCC ATGGGCGACT GGTTGTGGCC 1740

GATAGCGAAG GCAAGCTGTT TAGCGCCGCC ATTCCGAAGC AAGGGGATGG AAACGAACTG 1800

AAAATGAAAG CCATGCCTCA GCATGCGCTC GATGAACATT TTGGTCATGA CCACCAGATT 1860

TCTGGATTTT TCCATGACGA CCACGGCCAG CTTAATGCGC TGGTGAAAAA TAACTTCAGG 1920

CAGCAGCATG CCTGCCCGTT GGGTAACGAT CATCAGTTTC ACCCCGGCTG GAACCTGACT 1980

GATGCGCTGG TTATCGACAA TCAGCTGGGG CTGCATCATA CCAATCCTGA ACCGCATGAG 2040

ATTCTTGATA TGGGGCATTT AGGCAGCCTG GCGTTACAGG AGGGCAAGCT TCACTATTTT 2100

GACCAGCTGA CCAAAGGGTG GACTGGCGCG GAGTCAGATT GTAAGCAGCT GAAAAAAGGC 2160

CTGGATGGAG CAGCTTATCT ACTGAAAGAC GGTGAAGTGA AACGCCTGAA TATTAATCAG 2220

AGCACCTCCT CTATCAAGCA CGGAACGGAA AACGTTTTTT CGCTGCCGCA TGTGCGCAAT 2280

AAACCGGAGC CGGGAGATGC CCTGCAAGGG CTGAATAAAG ACGATAAGGC CCAGGCCATG 2340

GCGGTGATTG GGGTAAATAA ATACCTGGCG CTGACGGAAA AAGGGGACAT TCGCTCCTTC 2400

CAGATAAAAC CCGGCACCCA GCAGTTGGAG CGGCCGGCAC AAACTCTCAG CCGCGAAGGT 2460

ATCAGCGGCG AACTGAAAGA CATTCATGTC GACCACAAGC AGAACCTGTA TGCCTTGACC 2520

CACGAGGGAG AGGTGTTTCA TCAGCCGCGT GAAGCCTGGC AGAATGGTGC CGAAAGCAGC 2580

AGCTGGCACA AACTGGCGTT GCCACAGAGT GAAAGTAAGC TAAAAAGTCT GGACATGAGC 2640

CATGAGCACA AACCGATTGC CACCTTTGAA GACGGTAGCC AGCATCAGCT GAAGGCTGGC 2700

GGCTGGCACG CCTATGCGGC ACCTGAACGC GGGCCGCTGG CGGTGGGTAC CAGCGGTTCA 2760

CAAACCGTCT TTAACCGACT AATGCAGGGG GTGAAAGGCA AGGTGATCCC AGGCAGCGGG 2820

TTGACGGTTA AGCTCTCGGC TCAGACGGGG GGAATGACCG GCGCCGAAGG GCGCAAGGTC 2880

AGCAGTAAAT TTTCCGAAAG GATCCGCGCC TATGCGTTCA ACCCAACAAT GTCCACGCCG 2940

CGACCGATTA AAAATGCTGC TTATGCCACA CAGCACGGCT GGCAGGGGCG TGAGGGGTTG 3000

AAGCCGTTGT ACGAGATGCA GGGAGCGCTG ATTAAACAAC TGGATGCGCA TAACGTTCGT 3060

CATAACGCGC CACAGCCAGA TTTGCAGAGC AAACTGGAAA CTCTGGATTT AGGCGAACAT 3120

GGCGCAGAAT TGCTTAACGA CATGAAGCGC TTCCGCGACG AACTGGAGCA GAGTGCAACC 3180

CGTTCGGTGA CCGTTTTAGG TCAACATCAG GGAGTGCTAA AAAGCAACGG TGAAATCAAT 3240

AGCGAATTTA AGCCATCGCC CGGCAAGGCG TTGGTCCAGA GCTTTAACGT CAATCGCTCT 3300

GGTCAGGATC TAAGCAAGTC ACTGCAACAG GCAGTACATG CCACGCCGCC ATCCGCAGAG 3360

AGTAAACTGC AATCCATGCT GGGGCACTTT GTCAGTGCCG GGGTGGATAT GAGTCATCAG 3420

AAGGGCGAGA TCCCGCTGGG CCGCCAGCGC GATCCGAATG ATAAAACCGC ACTGACCAAA 3480

TCGCGTTTAA TTTTAGATAC CGTGACCATC GGTGAACTGC ATGAACTGGC CGATAAGGCG 3540

AAACTGGTAT CTGACCATAA ACCCGATGCC GATCAGATAA AACAGCTGCG CCAGCAGTTC 3600

GATACGCTGC GTGAAAAGCG GTATGAGAGC AATCCGGTGA AGCATTACAC CGATATGGGC 3660

TTCACCCATA ATAAGGCGCT GGAAGCAAAC TATGATGCGG TCAAAGCCTT TATCAATGCC 3720

TTTAAGAAAG AGCACCACGG CGTCAATCTG ACCACGCGTA CCGTACTGGA ATCACAGGGC 3780

AGTGCGGAGC TGGCGAAGAA GCTCAAGAAT ACGCTGTTGT CCCTGGACAG TGGTGAAAGT 3840

ATGAGCTTCA GCCGGTCATA TGGCGGGGGC GTCAGCACTG TCTTTGTGCC TACCCTTAGC 3900

AAGAAGGTGC CAGTTCCGGT GATCCCCGGA GCCGGCATCA CGCTGGATCG CGCCTATAAC 3960

CTGAGCTTCA GTCGTACCAG CGGCGGATTG AACGTCAGTT TTGGCCGCGA CGGCGGGGTG 4020

AGTGGTAACA TCATGGTCGC TACCGGCCAT GATGTGATGC CCTATATGAC CGGTAAGAAA 4080

ACCAGTGCAG GTAACGCCAG TGACTGGTTG AGCGCAAAAC ATAAAATCAG CCCGGACTTG 4140

CGTATCGGCG CTGCTGTGAG TGGCACCCTG CAAGGAACGC TACAAAACAG CCTGAAGTTT 4200

AAGCTGACAG AGGATGAGCT GCCTGGCTTT ATCCATGGCT TGACGCATGG CACGTTGACC 4260

CCGGCAGAAC TGTTGCAAAA GGGGATCGAA CATCAGATGA AGCAGGGCAG CAAACTGACG 4320

TTTAGCGTCG ATACCTCGGC AAATCTGGAT CTGCGTGCCG GTATCAATCT GAACGAAGAC 4380

GGCAGTAAAC CAAATGGTGT CACTGCCCGT GTTTCTGCCG GGCTAAGTGC ATCGGCAAAC 4440

CTGGCCGCCG GCTCGCGTGA ACGCAGCACC ACCTCTGGCC AGTTTGGCAG CACGACTTCG 4500

GCCAGCAATA ACCGCCCAAC CTTCCTCAAC GGGGTCGGCG CGGGTGCTAA CCTGACGGCT 4560

GCTTTAGGGG TTGCCCATTC ATCTACGCAT GAAGGGAAAC CGGTCGGGAT CTTCCCGGCA 4620

TTTACCTCGA CCAATGTTTC GGCAGCGCTG GCGCTGGATA ACCGTACCTC ACAGAGTATC 4680

AGCCTGGAAT TGAAGCGCGC GGAGCCGGTG ACCAGCAACG ATATCAGCGA GTTGACCTCC 4740

ACGCTGGGAA AACACTTTAA GGATAGCGCC ACAACGAAGA TGCTTGCCGC TCTCAAAGAG 4800

TTAGATGACG CTAAGCCCGC TGAACAACTG CATATTTTAC AGCAGCATTT CAGTGCAAAA 4860

GATGTCGTCG GTGATGAACG CTACGAGGCG GTGCGCAACC TGAAAAAACT GGTGATACGT 4920

CAACAGGCTG CGGACAGCCA CAGCATGGAA TTAGGATCTG CCAGTCACAG CACGACCTAC 4980

AATAATCTGT CGAGAATAAA TAATGACGGC ATTGTCGAGC TGCTACACAA ACATTTCGAT 5040

GCGGCATTAC CAGCAAGCAG TGCCAAACGT CTTGGTGAAA TGATGAATAA CGATCCGGCA 5100

```
CTGAAAGATA TTATTAAGCA GCTGCAAAGT ACGCCGTTCA GCAGCGCCAG CGTGTCGATG    5160

GAGCTGAAAG ATGGTCTGCG TGAGCAGACG GAAAAAGCAA TACTGGACGG TAAGGTCGGT    5220

CGTGAAGAAG TGGGAGTACT TTTCCAGGAT CGTAACAACT TGCGTGTTAA ATCGGTCAGC    5280

GTCAGTCAGT CCGTCAGCAA AAGCGAAGGC TTCAATACCC CAGCGCTGTT ACTGGGGACG    5340

AGCAACAGCG CTGCTATGAG CATGGAGCGC AACATCGGAA CCATTAATTT TAAATACGGC    5400

CAGGATCAGA ACACCCCACG GCGATTTACC CTGGAGGGTG GAATAGCTCA GGCTAATCCG    5460

CAGGTCGCAT CTGCGCTTAC TGATTTGAAG AAGGAAGGGC TGGAAATGAA GAGCTAA       5517
```

This DNA molecule is known as the dspE gene for *Erwinia amylovora*. This isolated DNA molecule of the present invention encodes a protein or polypeptide which elicits a plant pathogen's hypersensitive response having an amino acid sequence of SEQ. ID. No. 8 as follows:

```
Met Glu Leu Lys Ser Leu Gly Thr Glu His Lys Ala Ala Val His Thr
1               5                  10                  15

Ala Ala His Asn Pro Val Gly His Gly Val Ala Leu Gln Gln Gly Ser
            20                  25                  30

Ser Ser Ser Ser Pro Gln Asn Ala Ala Ala Ser Leu Ala Ala Glu Gly
        35                  40                  45

Lys Asn Arg Gly Lys Met Pro Arg Ile His Gln Pro Ser Thr Ala Ala
    50                  55                  60

Asp Gly Ile Ser Ala Ala His Gln Gln Lys Lys Ser Phe Ser Leu Arg
65                  70                  75                  80

Gly Cys Leu Gly Thr Lys Lys Phe Ser Arg Ser Ala Pro Gln Gly Gln
                85                  90                  95

Pro Gly Thr Thr His Ser Lys Gly Ala Thr Leu Arg Asp Leu Leu Ala
            100                 105                 110

Arg Asp Asp Gly Glu Thr Gln His Glu Ala Ala Ala Pro Asp Ala Ala
        115                 120                 125

Arg Leu Thr Arg Ser Gly Gly Val Lys Arg Arg Asn Met Asp Asp Met
    130                 135                 140

Ala Gly Arg Pro Met Val Lys Gly Gly Ser Gly Glu Asp Lys Val Pro
145                 150                 155                 160

Thr Gln Gln Lys Arg His Gln Leu Asn Asn Phe Gly Gln Met Arg Gln
                165                 170                 175

Thr Met Leu Ser Lys Met Ala His Pro Ala Ser Ala Asn Ala Gly Asp
            180                 185                 190

Arg Leu Gln His Ser Pro Pro His Ile Pro Gly Ser His His Glu Ile
        195                 200                 205
```

```
Lys Glu Glu Pro Val Gly Ser Thr Ser Lys Ala Thr Thr Ala His Ala
    210                 215                 220

Asp Arg Val Glu Ile Ala Gln Glu Asp Asp Asp Ser Glu Phe Gln Gln
225                 230                 235                 240

Leu His Gln Gln Arg Leu Ala Arg Glu Arg Glu Asn Pro Pro Gln Pro
                245                 250                 255

Pro Lys Leu Gly Val Ala Thr Pro Ile Ser Ala Arg Phe Gln Pro Lys
            260                 265                 270

Leu Thr Ala Val Ala Glu Ser Val Leu Glu Gly Thr Asp Thr Thr Gln
        275                 280                 285

Ser Pro Leu Lys Pro Gln Ser Met Leu Lys Gly Ser Gly Ala Gly Val
    290                 295                 300

Thr Pro Leu Ala Val Thr Leu Asp Lys Gly Lys Leu Gln Leu Ala Pro
305                 310                 315                 320

Asp Asn Pro Pro Ala Leu Asn Thr Leu Leu Lys Gln Thr Leu Gly Lys
                325                 330                 335

Asp Thr Gln His Tyr Leu Ala His His Ala Ser Ser Asp Gly Ser Gln
            340                 345                 350

His Leu Leu Leu Asp Asn Lys Gly His Leu Phe Asp Ile Lys Ser Thr
        355                 360                 365

Ala Thr Ser Tyr Ser Val Leu His Asn Ser His Pro Gly Glu Ile Lys
    370                 375                 380

Gly Lys Leu Ala Gln Ala Gly Thr Gly Ser Val Ser Val Asp Gly Lys
385                 390                 395                 400

Ser Gly Lys Ile Ser Leu Gly Ser Gly Thr Gln Ser His Asn Lys Thr
                405                 410                 415

Met Leu Ser Gln Pro Gly Glu Ala His Arg Ser Leu Leu Thr Gly Ile
            420                 425                 430

Trp Gln His Pro Ala Gly Ala Ala Arg Pro Gln Gly Glu Ser Ile Arg
        435                 440                 445

Leu His Asp Asp Lys Ile His Ile Leu His Pro Glu Leu Gly Val Trp
    450                 455                 460

Gln Ser Ala Asp Lys Asp Thr His Ser Gln Leu Ser Arg Gln Ala Asp
465                 470                 475                 480

Gly Lys Leu Tyr Ala Leu Lys Asp Asn Arg Thr Leu Gln Asn Leu Ser
                485                 490                 495

Asp Asn Lys Ser Ser Glu Lys Leu Val Asp Lys Ile Lys Ser Tyr Ser
            500                 505                 510

Val Asp Gln Arg Gly Gln Val Ala Ile Leu Thr Asp Thr Pro Gly Arg
        515                 520                 525

His Lys Met Ser Ile Met Pro Ser Leu Asp Ala Ser Pro Glu Ser His
    530                 535                 540

Ile Ser Leu Ser Leu His Phe Ala Asp Ala His Gln Gly Leu Leu His
545                 550                 555                 560
```

Gly Lys Ser Glu Leu Glu Ala Gln Ser Val Ala Ile Ser His Gly Arg
565 570 575

Leu Val Val Ala Asp Ser Glu Gly Lys Leu Phe Ser Ala Ala Ile Pro
580 585 590

Lys Gln Gly Asp Gly Asn Glu Leu Lys Met Lys Ala Met Pro Gln His
595 600 605

Ala Leu Asp Glu His Phe Gly His Asp His Gln Ile Ser Gly Phe Phe
610 615 620

His Asp Asp His Gly Gln Leu Asn Ala Leu Val Lys Asn Asn Phe Arg
625 630 635 640

Gln Gln His Ala Cys Pro Leu Gly Asn Asp His Gln Phe His Pro Gly
645 650 655

Trp Asn Leu Thr Asp Ala Leu Val Ile Asp Asn Gln Leu Gly Leu His
660 665 670

His Thr Asn Pro Glu Pro His Glu Ile Leu Asp Met Gly His Leu Gly
675 680 685

Ser Leu Ala Leu Gln Glu Gly Lys Leu His Tyr Phe Asp Gln Leu Thr
690 695 700

Lys Gly Trp Thr Gly Ala Glu Ser Asp Cys Lys Gln Leu Lys Lys Gly
705 710 715 720

Leu Asp Gly Ala Ala Tyr Leu Leu Lys Asp Gly Glu Val Lys Arg Leu
725 730 735

Asn Ile Asn Gln Ser Thr Ser Ser Ile Lys His Gly Thr Glu Asn Val
740 745 750

Phe Ser Leu Pro His Val Arg Asn Lys Pro Glu Pro Gly Asp Ala Leu
755 760 765

Gln Gly Leu Asn Lys Asp Asp Lys Ala Gln Ala Met Ala Val Ile Gly
770 775 780

Val Asn Lys Tyr Leu Ala Leu Thr Glu Lys Gly Asp Ile Arg Ser Phe
785 790 795 800

Gln Ile Lys Pro Gly Thr Gln Gln Leu Glu Arg Pro Ala Gln Thr Leu
805 810 815

Ser Arg Glu Gly Ile Ser Gly Glu Leu Lys Asp Ile His Val Asp His
820 825 830

Lys Gln Asn Leu Tyr Ala Leu Thr His Glu Gly Glu Val Phe His Gln
835 840 845

Pro Arg Glu Ala Trp Gln Asn Gly Ala Glu Ser Ser Ser Trp His Lys
850 855 860

Leu Ala Leu Pro Gln Ser Glu Ser Lys Leu Lys Ser Leu Asp Met Ser
865 870 875 880

His Glu His Lys Pro Ile Ala Thr Phe Glu Asp Gly Ser Gln His Gln
885 890 895

Leu Lys Ala Gly Gly Trp His Ala Tyr Ala Ala Pro Glu Arg Gly Pro
900 905 910

Leu Ala Val Gly Thr Ser Gly Ser Gln Thr Val Phe Asn Arg Leu Met
915 920 925

Gln Gly Val Lys Gly Lys Val Ile Pro Gly Ser Gly Leu Thr Val Lys
930 935 940

Leu Ser Ala Gln Thr Gly Gly Met Thr Gly Ala Glu Gly Arg Lys Val
945 950 955 960

Ser Ser Lys Phe Ser Glu Arg Ile Arg Ala Tyr Ala Phe Asn Pro Thr
965 970 975

Met Ser Thr Pro Arg Pro Ile Lys Asn Ala Ala Tyr Ala Thr Gln His
980 985 990

Gly Trp Gln Gly Arg Glu Gly Leu Lys Pro Leu Tyr Glu Met Gln Gly
995 1000 1005

Ala Leu Ile Lys Gln Leu Asp Ala His Asn Val Arg His Asn Ala Pro
1010 1015 1020

Gln Pro Asp Leu Gln Ser Lys Leu Glu Thr Leu Asp Leu Gly Glu His
1025 1030 1035 1040

Gly Ala Glu Leu Leu Asn Asp Met Lys Arg Phe Arg Asp Glu Leu Glu
1045 1050 1055

Gln Ser Ala Thr Arg Ser Val Thr Val Leu Gly Gln His Gln Gly Val
1060 1065 1070

Leu Lys Ser Asn Gly Glu Ile Asn Ser Glu Phe Lys Pro Ser Pro Gly
1075 1080 1085

Lys Ala Leu Val Gln Ser Phe Asn Val Asn Arg Ser Gly Gln Asp Leu
1090 1095 1100

Ser Lys Ser Leu Gln Gln Ala Val His Ala Thr Pro Pro Ser Ala Glu
1105 1110 1115 1120

Ser Lys Leu Gln Ser Met Leu Gly His Phe Val Ser Ala Gly Val Asp
1125 1130 1135

Met Ser His Gln Lys Gly Glu Ile Pro Leu Gly Arg Gln Arg Asp Pro
1140 1145 1150

Asn Asp Lys Thr Ala Leu Thr Lys Ser Arg Leu Ile Leu Asp Thr Val
1155 1160 1165

Thr Ile Gly Glu Leu His Glu Leu Ala Asp Lys Ala Lys Leu Val Ser
1170 1175 1180

Asp His Lys Pro Asp Ala Asp Gln Ile Lys Gln Leu Arg Gln Gln Phe
1185 1190 1195 1200

Asp Thr Leu Arg Glu Lys Arg Tyr Glu Ser Asn Pro Val Lys His Tyr
1205 1210 1215

Thr Asp Met Gly Phe Thr His Asn Lys Ala Leu Glu Ala Asn Tyr Asp
1220 1225 1230

Ala Val Lys Ala Phe Ile Asn Ala Phe Lys Lys Glu His His Gly Val
1235 1240 1245

Asn Leu Thr Thr Arg Thr Val Leu Glu Ser Gln Gly Ser Ala Glu Leu
1250 1255 1260

Ala Lys Lys Leu Lys Asn Thr Leu Leu Ser Leu Asp Ser Gly Glu Ser
1265 1270 1275 1280

Met Ser Phe Ser Arg Ser Tyr Gly Gly Gly Val Ser Thr Val Phe Val
1285 1290 1295

Pro Thr Leu Ser Lys Lys Val Pro Val Pro Val Ile Pro Gly Ala Gly
1300 1305 1310

Ile Thr Leu Asp Arg Ala Tyr Asn Leu Ser Phe Ser Arg Thr Ser Gly
1315 1320 1325

Gly Leu Asn Val Ser Phe Gly Arg Asp Gly Gly Val Ser Gly Asn Ile
1330 1335 1340

Met Val Ala Thr Gly His Asp Val Met Pro Tyr Met Thr Gly Lys Lys
1345 1350 1355 1360

Thr Ser Ala Gly Asn Ala Ser Asp Trp Leu Ser Ala Lys His Lys Ile
1365 1370 1375

Ser Pro Asp Leu Arg Ile Gly Ala Ala Val Ser Gly Thr Leu Gln Gly
1380 1385 1390

Thr Leu Gln Asn Ser Leu Lys Phe Lys Leu Thr Glu Asp Glu Leu Pro
1395 1400 1405

Gly Phe Ile His Gly Leu Thr His Gly Thr Leu Thr Pro Ala Glu Leu
1410 1415 1420

Leu Gln Lys Gly Ile Glu His Gln Met Lys Gln Gly Ser Lys Leu Thr
1425 1430 1435 1440

Phe Ser Val Asp Thr Ser Ala Asn Leu Asp Leu Arg Ala Gly Ile Asn
1445 1450 1455

Leu Asn Glu Asp Gly Ser Lys Pro Asn Gly Val Thr Ala Arg Val Ser
1460 1465 1470

Ala Gly Leu Ser Ala Ser Ala Asn Leu Ala Ala Gly Ser Arg Glu Arg
1475 1480 1485

Ser Thr Thr Ser Gly Gln Phe Gly Ser Thr Thr Ser Ala Ser Asn Asn
1490 1495 1500

Arg Pro Thr Phe Leu Asn Gly Val Gly Ala Gly Ala Asn Leu Thr Ala
1505 1510 1515 1520

Ala Leu Gly Val Ala His Ser Ser Thr His Glu Gly Lys Pro Val Gly
1525 1530 1535

Ile Phe Pro Ala Phe Thr Ser Thr Asn Val Ser Ala Ala Leu Ala Leu
1540 1545 1550

Asp Asn Arg Thr Ser Gln Ser Ile Ser Leu Glu Leu Lys Arg Ala Glu
1555 1560 1565

Pro Val Thr Ser Asn Asp Ile Ser Glu Leu Thr Ser Thr Leu Gly Lys
1570 1575 1580

```
His Phe Lys Asp Ser Ala Thr Thr Lys Met Leu Ala Ala Leu Lys Glu
1585                1590                1595                1600

Leu Asp Asp Ala Lys Pro Ala Glu Gln Leu His Ile Leu Gln Gln His
                1605                1610                1615

Phe Ser Ala Lys Asp Val Val Gly Asp Glu Arg Tyr Glu Ala Val Arg
            1620                1625                1630

Asn Leu Lys Lys Leu Val Ile Arg Gln Gln Ala Ala Asp Ser His Ser
        1635                1640                1645

Met Glu Leu Gly Ser Ala Ser His Ser Thr Thr Tyr Asn Asn Leu Ser
    1650                1655                1660

Arg Ile Asn Asn Asp Gly Ile Val Glu Leu Leu His Lys His Phe Asp
1665                1670                1675                1680

Ala Ala Leu Pro Ala Ser Ser Ala Lys Arg Leu Gly Glu Met Met Asn
                1685                1690                1695

Asn Asp Pro Ala Leu Lys Asp Ile Ile Lys Gln Leu Gln Ser Thr Pro
            1700                1705                1710

Phe Ser Ser Ala Ser Val Ser Met Glu Leu Lys Asp Gly Leu Arg Glu
        1715                1720                1725

Gln Thr Glu Lys Ala Ile Leu Asp Gly Lys Val Gly Arg Glu Glu Val
    1730                1735                1740

Gly Val Leu Phe Gln Asp Arg Asn Asn Leu Arg Val Lys Ser Val Ser
1745                1750                1755                1760

Val Ser Gln Ser Val Ser Lys Ser Glu Gly Phe Asn Thr Pro Ala Leu
                1765                1770                1775

Leu Leu Gly Thr Ser Asn Ser Ala Ala Met Ser Met Glu Arg Asn Ile
            1780                1785                1790

Gly Thr Ile Asn Phe Lys Tyr Gly Gln Asp Gln Asn Thr Pro Arg Arg
        1795                1800                1805

Phe Thr Leu Glu Gly Gly Ile Ala Gln Ala Asn Pro Gln Val Ala Ser
    1810                1815                1820

Ala Leu Thr Asp Leu Lys Lys Glu Gly Leu Glu Met Lys Ser
1825                1830                1835
```

This protein or polypeptide is about 198 kDa and has a pI of 8.98.

The present invention relates to an isolated DNA molecule having a nucleotide sequence of SEQ. ID. No. 9 as follows:

```
ATGACATCGT CACAGCAGCG GGTTGAAAGG TTTTTACAGT ATTTCTCCGC CGGGTGTAAA      60

ACGCCCATAC ATCTGAAAGA CGGGGTGTGC GCCCTGTATA ACGAACAAGA TGAGGAGGCG     120

GCGGTGCTGG AAGTACCGCA ACACAGCGAC AGCCTGTTAC TACACTGCCG AATCATTGAG     180

GCTGACCCAC AAACTTCAAT AACCCTGTAT TCGATGCTAT TACAGCTGAA TTTTGAAATG     240
```

```
GCGGCCATGC GCGGCTGTTG GCTGGCGCTG GATGAACTGC ACAACGTGCG TTTATGTTTT     300

CAGCAGTCGC TGGAGCATCT GGATGAAGCA AGTTTTAGCG ATATCGTTAG CGGCTTCATC     360

GAACATGCGG CAGAAGTGCG TGAGTATATA GCGCAATTAG ACGAGAGTAG CGCGGCATAA     420
```

This is known as the dspF gene. This isolated DNA molecule of the present invention encodes a hypersensitive response elicitor protein or polypeptide having an amino acid sequence of SEQ. ID. No. 10 as follows:

```
Met Thr Ser Ser Gln Gln Arg Val Glu Arg Phe Leu Gln Tyr Phe Ser
1               5                  10                  15

Ala Gly Cys Lys Thr Pro Ile His Leu Lys Asp Gly Val Cys Ala Leu
            20                  25                  30

Tyr Asn Glu Gln Asp Glu Glu Ala Ala Val Leu Glu Val Pro Gln His
        35                  40                  45

Ser Asp Ser Leu Leu Leu His Cys Arg Ile Ile Glu Ala Asp Pro Gln
    50                  55                  60

Thr Ser Ile Thr Leu Tyr Ser Met Leu Leu Gln Leu Asn Phe Glu Met
65                  70                  75                  80

Ala Ala Met Arg Gly Cys Trp Leu Ala Leu Asp Glu Leu His Asn Val
                85                  90                  95

Arg Leu Cys Phe Gln Gln Ser Leu Glu His Leu Asp Glu Ala Ser Phe
            100                 105                 110

Ser Asp Ile Val Ser Gly Phe Ile Glu His Ala Ala Glu Val Arg Glu
        115                 120                 125

Tyr Ile Ala Gln Leu Asp Glu Ser Ser Ala Ala
    130                 135
```

This protein or polypeptide is about 16 kDa and has a pI of 4.45.

The hypersensitive response elicitor polypeptide or protein derived from *Pseudomonas syringae* has an amino acid sequence corresponding to SEQ. ID. No. 11 as follows:

```
Met Gln Ser Leu Ser Leu Asn Ser Ser Ser Leu Gln Thr Pro Ala Met
1               5                  10                  15

Ala Leu Val Leu Val Arg Pro Glu Ala Glu Thr Thr Gly Ser Thr Ser
            20                  25                  30

Ser Lys Ala Leu Gln Glu Val Val Val Lys Leu Ala Glu Glu Leu Met
        35                  40                  45

Arg Asn Gly Gln Leu Asp Asp Ser Ser Pro Leu Gly Lys Leu Leu Ala
    50                  55                  60
```

Lys Ser Met Ala Ala Asp Gly Lys Ala Gly Gly Gly Ile Glu Asp Val
65 70 75 80

Ile Ala Ala Leu Asp Lys Leu Ile His Glu Lys Leu Gly Asp Asn Phe
85 90 95

Gly Ala Ser Ala Asp Ser Ala Ser Gly Thr Gly Gln Gln Asp Leu Met
100 105 110

Thr Gln Val Leu Asn Gly Leu Ala Lys Ser Met Leu Asp Asp Leu Leu
115 120 125

Thr Lys Gln Asp Gly Gly Thr Ser Phe Ser Glu Asp Asp Met Pro Met
130 135 140

Leu Asn Lys Ile Ala Gln Phe Met Asp Asp Asn Pro Ala Gln Phe Pro
145 150 155 160

Lys Pro Asp Ser Gly Ser Trp Val Asn Glu Leu Lys Glu Asp Asn Phe
165 170 175

Leu Asp Gly Asp Glu Thr Ala Ala Phe Arg Ser Ala Leu Asp Ile Ile
180 185 190

Gly Gln Gln Leu Gly Asn Gln Gln Ser Asp Ala Gly Ser Leu Ala Gly
195 200 205

Thr Gly Gly Gly Leu Gly Thr Pro Ser Ser Phe Ser Asn Asn Ser Ser
210 215 220

Val Met Gly Asp Pro Leu Ile Asp Ala Asn Thr Gly Pro Gly Asp Ser
225 230 235 240

Gly Asn Thr Arg Gly Glu Ala Gly Gln Leu Ile Gly Glu Leu Ile Asp
245 250 255

Arg Gly Leu Gln Ser Val Leu Ala Gly Gly Gly Leu Gly Thr Pro Val
260 265 270

Asn Thr Pro Gln Thr Gly Thr Ser Ala Asn Gly Gly Gln Ser Ala Gln
275 280 285

Asp Leu Asp Gln Leu Leu Gly Gly Leu Leu Leu Lys Gly Leu Glu Ala
290 295 300

Thr Leu Lys Asp Ala Gly Gln Thr Gly Thr Asp Val Gln Ser Ser Ala
305 310 315 320

Ala Gln Ile Ala Thr Leu Leu Val Ser Thr Leu Leu Gln Gly Thr Arg
325 330 335

Asn Gln Ala Ala Ala
340

This hypersensitive response elicitor polypeptide or protein has a molecular weight of 34-35 kDa. It is rich in glycine (about 13.5%) and lacks cysteine and tyrosine. Further information about the hypersensitive response elicitor derived from *Pseudomonas syringae* is found in He, S. Y., H. C. Huang, and A. Collmer, "*Pseudomonas syringae* pv. *syringae* $Harpin_{Pss}$: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266 (1993), which is hereby incorporated by reference. The DNA molecule encoding the hypersensitive response elicitor from *Pseudomonas syringae* has a nucleotide sequence corresponding to SEQ. ID. No. 12 as follows:

```
ATGCAGAGTC TCAGTCTTAA CAGCAGCTCG CTGCAAACCC CGGCAATGGC CCTTGTCCTG      60
GTACGTCCTG AAGCCGAGAC GACTGGCAGT ACGTCGAGCA AGGCGCTTCA GGAAGTTGTC     120
GTGAAGCTGG CCGAGGAACT GATGCGCAAT GGTCAACTCG ACGACAGCTC GCCATTGGGA     180
AAACTGTTGG CCAAGTCGAT GGCCGCAGAT GGCAAGGCGG GCGGCGGTAT TGAGGATGTC     240
ATCGCTGCGC TGGACAAGCT GATCCATGAA AAGCTCGGTG ACAACTTCGG CGCGTCTGCG     300
GACAGCGCCT CGGGTACCGG ACAGCAGGAC CTGATGACTC AGGTGCTCAA TGGCCTGGCC     360
AAGTCGATGC TCGATGATCT TCTGACCAAG CAGGATGGCG GGACAAGCTT CTCCGAAGAC     420
GATATGCCGA TGCTGAACAA GATCGCGCAG TTCATGGATG ACAATCCCGC ACAGTTTCCC     480
AAGCCGGACT CGGGCTCCTG GGTGAACGAA CTCAAGGAAG ACAACTTCCT TGATGGCGAC     540
GAAACGGCTG CGTTCCGTTC GGCACTCGAC ATCATTGGCC AGCAACTGGG TAATCAGCAG     600
AGTGACGCTG GCAGTCTGGC AGGGACGGGT GGAGGTCTGG GCACTCCGAG CAGTTTTTCC     660
AACAACTCGT CCGTGATGGG TGATCCGCTG ATCGACGCCA ATACCGGTCC CGGTGACAGC     720
GGCAATACCC GTGGTGAAGC GGGGCAACTG ATCGGCGAGC TTATCGACCG TGGCCTGCAA     780
TCGGTATTGG CCGGTGGTGG ACTGGGCACA CCCGTAAACA CCCCGCAGAC CGGTACGTCG     840
GCGAATGGCG GACAGTCCGC TCAGGATCTT GATCAGTTGC TGGGCGGCTT GCTGCTCAAG     900
GGCCTGGAGG CAACGCTCAA GGATGCCGGG CAAACAGGCA CCGACGTGCA GTCGAGCGCT     960
GCGCAAATCG CCACCTTGCT GGTCAGTACG CTGCTGCAAG GCACCCGCAA TCAGGCTGCA    1020
GCCTGA                                                               1026
```

Another potentially suitable hypersensitive response elicitor from *Pseudomonas syringae* is disclosed in U.S. Patent Application Serial No. 09/120,817,

which is hereby incorporated by reference. The protein has a nucleotide sequence of SEQ. ID. No. 13 as follows:

TCCACTTCGC TGATTTTGAA ATTGGCAGAT TCATAGAAAC GTTCAGGTGT GGAAATCAGG 60

CTGAGTGCGC AGATTTCGTT GATAAGGGTG TGGTACTGGT CATTGTTGGT CATTTCAAGG 120

CCTCTGAGTG CGGTGCGGAG CAATACCAGT CTTCCTGCTG GCGTGTGCAC ACTGAGTCGC 180

AGGCATAGGC ATTTCAGTTC CTTGCGTTGG TTGGGCATAT AAAAAAAGGA ACTTTTAAAA 240

ACAGTGCAAT GAGATGCCGG CAAAACGGGA ACCGGTCGCT GCGCTTTGCC ACTCACTTCG 300

AGCAAGCTCA ACCCCAAACA TCCACATCCC TATCGAACGG ACAGCGATAC GGCCACTTGC 360

TCTGGTAAAC CCTGGAGCTG GCGTCGGTCC AATTGCCCAC TTAGCGAGGT AACGCAGCAT 420

GAGCATCGGC ATCACACCCC GGCCGCAACA GACCACCACG CCACTCGATT TTTCGGCGCT 480

AAGCGGCAAG AGTCCTCAAC CAAACACGTT CGGCGAGCAG AACACTCAGC AAGCGATCGA 540

CCCGAGTGCA CTGTTGTTCG GCAGCGACAC ACAGAAAGAC GTCAACTTCG GCACGCCCGA 600

CAGCACCGTC CAGAATCCGC AGGACGCCAG CAAGCCCAAC GACAGCCAGT CCAACATCGC 660

TAAATTGATC AGTGCATTGA TCATGTCGTT GCTGCAGATG CTCACCAACT CCAATAAAAA 720

GCAGGACACC AATCAGGAAC AGCCTGATAG CCAGGCTCCT TTCCAGAACA ACGGCGGGCT 780

CGGTACACCG TCGGCCGATA GCGGGGGCGG CGGTACACCG GATGCGACAG GTGGCGGCGG 840

CGGTGATACG CCAAGCGCAA CAGGCGGTGG CGGCGGTGAT ACTCCGACCG CAACAGGCGG 900

TGGCGGCAGC GGTGGCGGCG GCACACCCAC TGCAACAGGT GGCGGCAGCG GTGGCACACC 960

CACTGCAACA GGCGGTGGCG AGGGTGGCGT AACACCGCAA ATCACTCCGC AGTTGGCCAA 1020

CCCTAACCGT ACCTCAGGTA CTGGCTCGGT GTCGGACACC GCAGGTTCTA CCGAGCAAGC 1080

CGGCAAGATC AATGTGGTGA AAGACACCAT CAAGGTCGGC GCTGGCGAAG TCTTTGACGG 1140

CCACGGCGCA ACCTTCACTG CCGACAAATC TATGGGTAAC GGAGACCAGG GCGAAAATCA 1200

GAAGCCCATG TTCGAGCTGG CTGAAGGCGC TACGTTGAAG AATGTGAACC TGGGTGAGAA 1260

CGAGGTCGAT GGCATCCACG TGAAAGCCAA AAACGCTCAG GAAGTCACCA TTGACAACGT 1320

GCATGCCCAG AACGTCGGTG AAGACCTGAT TACGGTCAAA GGCGAGGGAG GCGCAGCGGT 1380

CACTAATCTG AACATCAAGA ACAGCAGTGC CAAAGGTGCA GACGACAAGG TTGTCCAGCT 1440

CAACGCCAAC ACTCACTTGA AAATCGACAA CTTCAAGGCC GACGATTTCG GCACGATGGT 1500

TCGCACCAAC GGTGGCAAGC AGTTTGATGA CATGAGCATC GAGCTGAACG GCATCGAAGC 1560

TAACCACGGC AAGTTCGCCC TGGTGAAAAG CGACAGTGAC GATCTGAAGC TGGCAACGGG 1620

CAACATCGCC ATGACCGACG TCAAACACGC CTACGATAAA ACCCAGGCAT CGACCCAACA 1680

CACCGAGCTT TGAATCCAGA CAAGTAGCTT GAAAAAAGGG GGTGGACTC 1729

This DNA molecule is known as the dspE gene for *Pseudomonas syringae*. This isolated DNA molecule of the present invention encodes a protein or polypeptide which elicits a plant pathogen's hypersensitive response having an amino acid sequence of SEQ. ID. No. 14 as follows:

```
Met Ser Ile Gly Ile Thr Pro Arg Pro Gln Gln Thr Thr Thr Pro Leu
1               5                  10                  15

Asp Phe Ser Ala Leu Ser Gly Lys Ser Pro Gln Pro Asn Thr Phe Gly
            20                  25                  30

Glu Gln Asn Thr Gln Gln Ala Ile Asp Pro Ser Ala Leu Leu Phe Gly
        35                  40                  45

Ser Asp Thr Gln Lys Asp Val Asn Phe Gly Thr Pro Asp Ser Thr Val
    50                  55                  60

Gln Asn Pro Gln Asp Ala Ser Lys Pro Asn Asp Ser Gln Ser Asn Ile
65                  70                  75                  80

Ala Lys Leu Ile Ser Ala Leu Ile Met Ser Leu Leu Gln Met Leu Thr
                85                  90                  95

Asn Ser Asn Lys Lys Gln Asp Thr Asn Gln Glu Gln Pro Asp Ser Gln
            100                 105                 110

Ala Pro Phe Gln Asn Asn Gly Gly Leu Gly Thr Pro Ser Ala Asp Ser
        115                 120                 125

Gly Gly Gly Gly Thr Pro Asp Ala Thr Gly Gly Gly Gly Gly Asp Thr
    130                 135                 140

Pro Ser Ala Thr Gly Gly Gly Gly Gly Asp Thr Pro Thr Ala Thr Gly
145                 150                 155                 160

Gly Gly Gly Ser Gly Gly Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly
                165                 170                 175

Ser Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly Glu Gly Gly Val Thr
            180                 185                 190

Pro Gln Ile Thr Pro Gln Leu Ala Asn Pro Asn Arg Thr Ser Gly Thr
        195                 200                 205

Gly Ser Val Ser Asp Thr Ala Gly Ser Thr Glu Gln Ala Gly Lys Ile
    210                 215                 220

Asn Val Val Lys Asp Thr Ile Lys Val Gly Ala Gly Glu Val Phe Asp
225                 230                 235                 240

Gly His Gly Ala Thr Phe Thr Ala Asp Lys Ser Met Gly Asn Gly Asp
                245                 250                 255

Gln Gly Glu Asn Gln Lys Pro Met Phe Glu Leu Ala Glu Gly Ala Thr
            260                 265                 270
```

```
Leu Lys Asn Val Asn Leu Gly Glu Asn Glu Val Asp Gly Ile His Val
        275                 280                 285

Lys Ala Lys Asn Ala Gln Glu Val Thr Ile Asp Asn Val His Ala Gln
    290                 295                 300

Asn Val Gly Glu Asp Leu Ile Thr Val Lys Gly Glu Gly Gly Ala Ala
305                 310                 315                 320

Val Thr Asn Leu Asn Ile Lys Asn Ser Ser Ala Lys Gly Ala Asp Asp
                325                 330                 335

Lys Val Val Gln Leu Asn Ala Asn Thr His Leu Lys Ile Asp Asn Phe
            340                 345                 350

Lys Ala Asp Asp Phe Gly Thr Met Val Arg Thr Asn Gly Gly Lys Gln
        355                 360                 365

Phe Asp Asp Met Ser Ile Glu Leu Asn Gly Ile Glu Ala Asn His Gly
    370                 375                 380

Lys Phe Ala Leu Val Lys Ser Asp Ser Asp Asp Leu Lys Leu Ala Thr
385                 390                 395                 400

Gly Asn Ile Ala Met Thr Asp Val Lys His Ala Tyr Asp Lys Thr Gln
                405                 410                 415

Ala Ser Thr Gln His Thr Glu Leu
            420
```

This protein or polypeptide is about 42.9 kDa.

This hypersensitive response elicitor from *Pseudomonas syringae* has 1 hypersensitive response eliciting domain. This domain extends, within SEQ. ID. No. 14, from amino acid 45 to amino acid 102, particularly from amino acid 58 to amino acid 92. The acidic unit in the first domain extends, within SEQ. ID. No. 14, from amino acid 45 to amino acid 79, particularly from amino acid 58 to amino acid 79. The alpha-helix in the first domain extends, within SEQ. ID. No. 14, from amino acid 79 to amino acid 102, particularly from amino acid 79 to amino acid 92.

The hypersensitive response elicitor polypeptide or protein derived from *Pseudomonas solanacearum* has an amino acid sequence corresponding to SEQ. ID. No. 15 as follows:

```
Met Ser Val Gly Asn Ile Gln Ser Pro Ser Asn Leu Pro Gly Leu Gln
1               5                   10                  15

Asn Leu Asn Leu Asn Thr Asn Thr Asn Ser Gln Gln Ser Gly Gln Ser
            20                  25                  30
```

Val Gln Asp Leu Ile Lys Gln Val Glu Lys Asp Ile Leu Asn Ile Ile
35 40 45

Ala Ala Leu Val Gln Lys Ala Ala Gln Ser Ala Gly Gly Asn Thr Gly
50 55 60

Asn Thr Gly Asn Ala Pro Ala Lys Asp Gly Asn Ala Asn Ala Gly Ala
65 70 75 80

Asn Asp Pro Ser Lys Asn Asp Pro Ser Lys Ser Gln Ala Pro Gln Ser
85 90 95

Ala Asn Lys Thr Gly Asn Val Asp Asp Ala Asn Asn Gln Asp Pro Met
100 105 110

Gln Ala Leu Met Gln Leu Leu Glu Asp Leu Val Lys Leu Leu Lys Ala
115 120 125

Ala Leu His Met Gln Gln Pro Gly Gly Asn Asp Lys Gly Asn Gly Val
130 135 140

Gly Gly Ala Asn Gly Ala Lys Gly Ala Gly Gly Gln Gly Gly Leu Ala
145 150 155 160

Glu Ala Leu Gln Glu Ile Glu Gln Ile Leu Ala Gln Leu Gly Gly Gly
165 170 175

Gly Ala Gly Ala Gly Gly Ala Gly Gly Gly Val Gly Gly Ala Gly Gly
180 185 190

Ala Asp Gly Gly Ser Gly Ala Gly Gly Ala Gly Gly Ala Asn Gly Ala
195 200 205

Asp Gly Gly Asn Gly Val Asn Gly Asn Gln Ala Asn Gly Pro Gln Asn
210 215 220

Ala Gly Asp Val Asn Gly Ala Asn Gly Ala Asp Asp Gly Ser Glu Asp
225 230 235 240

Gln Gly Gly Leu Thr Gly Val Leu Gln Lys Leu Met Lys Ile Leu Asn
245 250 255

Ala Leu Val Gln Met Met Gln Gln Gly Gly Leu Gly Gly Gly Asn Gln
260 265 270

Ala Gln Gly Gly Ser Lys Gly Ala Gly Asn Ala Ser Pro Ala Ser Gly
275 280 285

Ala Asn Pro Gly Ala Asn Gln Pro Gly Ser Ala Asp Asp Gln Ser Ser
290 295 300

Gly Gln Asn Asn Leu Gln Ser Gln Ile Met Asp Val Val Lys Glu Val
305 310 315 320

Val Gln Ile Leu Gln Gln Met Leu Ala Ala Gln Asn Gly Gly Ser Gln
325 330 335

Gln Ser Thr Ser Thr Gln Pro Met
340

It is encoded by a DNA molecule having a nucleotide sequence corresponding SEQ. ID. No. 16 as follows:

```
ATGTCAGTCG GAAACATCCA GAGCCCGTCG AACCTCCCGG GTCTGCAGAA CCTGAACCTC      60
AACACCAACA CCAACAGCCA GCAATCGGGC CAGTCCGTGC AAGACCTGAT CAAGCAGGTC     120
GAGAAGGACA TCCTCAACAT CATCGCAGCC CTCGTGCAGA AGGCCGCACA GTCGGCGGGC     180
GGCAACACCG GTAACACCGG CAACGCGCCG GCGAAGGACG GCAATGCCAA CGCGGGCGCC     240
AACGACCCGA GCAAGAACGA CCCGAGCAAG AGCCAGGCTC CGCAGTCGGC CAACAAGACC     300
GGCAACGTCG ACGACGCCAA CAACCAGGAT CCGATGCAAG CGCTGATGCA GCTGCTGGAA     360
GACCTGGTGA AGCTGCTGAA GGCGGCCCTG CACATGCAGC AGCCCGGCGG CAATGACAAG     420
GGCAACGGCG TGGGCGGTGC CAACGGCGCC AAGGGTGCCG GCGGCCAGGG CGGCCTGGCC     480
GAAGCGCTGC AGGAGATCGA GCAGATCCTC GCCCAGCTCG GCGGCGGCGG TGCTGGCGCC     540
GGCGGCGCGG GTGGCGGTGT CGGCGGTGCT GGTGGCGCGG ATGGCGGCTC CGGTGCGGGT     600
GGCGCAGGCG GTGCGAACGG CGCCGACGGC GGCAATGGCG TGAACGGCAA CCAGGCGAAC     660
GGCCCGCAGA ACGCAGGCGA TGTCAACGGT GCCAACGGCG CGGATGACGG CAGCGAAGAC     720
CAGGGCGGCC TCACCGGCGT GCTGCAAAAG CTGATGAAGA TCCTGAACGC GCTGGTGCAG     780
ATGATGCAGC AAGGCGGCCT CGGCGGCGGC AACCAGGCGC AGGGCGGCTC GAAGGGTGCC     840
GGCAACGCCT CGCCGGCTTC CGGCGCGAAC CCGGGCGCGA ACCAGCCCGG TTCGGCGGAT     900
GATCAATCGT CCGGCCAGAA CAATCTGCAA TCCCAGATCA TGGATGTGGT GAAGGAGGTC     960
GTCCAGATCC TGCAGCAGAT GCTGGCGGCG CAGAACGGCG GCAGCCAGCA GTCCACCTCG    1020
ACGCAGCCGA TGTAA                                                     1035
```

Further information regarding the hypersensitive response elicitor polypeptide or protein derived from *Pseudomonas solanacearum* is set forth in Arlat, M., F. Van Gijsegem, J. C. Huet, J. C. Pemollet, and C. A. Boucher, "PopA1, a Protein which Induces a Hypersensitive-like Response in Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-533 (1994), which is hereby incorporated by reference.

The hypersensitive response elicitor from *Pseudomonas solanacearum* has 2 hypersensitive response eliciting domains. The first domain extends, within SEQ. ID.

No. 15, from amino acid 85 to amino acid 131, particularly from amino acid 95 to amino acid 123. The acidic unit in the first domain extends, within SEQ. ID. No. 15, from amino acid 85 to amino acid 111, particularly from amino acid 95 to amino acid 123. The alpha-helix in the first domain extends, within SEQ. ID. No. 15, from amino acid 85 to amino acid 111, particularly from amino acid 95 to amino acid 111. The second domain extends, within SEQ. ID. No. 15, from amino acid 195 to amino acid 264, particularly from amino acid 229 to amino acid 258. The acidic unit in the second domain extends, within SEQ. ID. No. 15, from amino acid 195 to amino acid 246, particularly from amino acid 229 to amino acid 264. The alpha-helix in the second domain extends, within SEQ. ID. No. 15, from amino acid 246 to amino acid 264, particularly from amino acid 246 to amino acid 258.

The N-terminus of the hypersensitive response elicitor polypeptide or protein from *Xanthomonas campestris* has an amino acid sequence corresponding to SEQ. ID. No. 17 as follows:

```
Met Asp Gly Ile Gly Asn His Phe Ser Asn
  1               5                  10
```

The hypersensitive response elicitor polypeptide or protein from *Xanthomonas campestris pv. pelargonii* is heat stable, protease sensitive, and has a molecular weight of 20 kDa. It includes an amino acid sequence corresponding to SEQ. ID. No. 18 as follows:

```
Ser Ser Gln Gln Ser Pro Ser Ala Gly Ser Glu Gln Gln Leu Asp Gln
1               5                  10                  15

Leu Leu Ala Met
            20
```

Isolation of *Erwinia carotovora* hypersensitive response elictor protein or polypeptide is described in Cui et al., "The RsmA Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrp* N_{Ecc} and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI, 9(7):565-73 (1996), which is hereby incorporated by reference. The hypersensitive response elicitor protein or polypeptide of *Erwinia stewartii* is set forth in Ahmad et al., "Harpin is Not

Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microbe Interact., July 14-19, 1996 and Ahmad, et al., "Harpin is Not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," Ann. Mtg. Am. Phytopath. Soc., July 27-31, 1996, which are hereby incorporated by reference.

Hypersensitive response elicitor proteins or polypeptides from *Phytophthora parasitica, Phytophthora cryptogea, Phytophthora cinnamoni, Phytophthora capsici, Phytophthora megasperma,* and *Phytophora citrophthora* are described in Kaman, et al., "Extracellular Protein Elicitors from Phytophthora: Most Specificity and Induction of Resistance to Bacterial and Fungal Phytopathogens," Molec. Plant-Microbe Interact., 6(1):15-25 (1993), Ricci et al., "Structure and Activity of Proteins from Pathogenic Fungi Phytophthora Eliciting Necrosis and Acquired Resistance in Tobacco," Eur. J. Biochem., 183:555-63 (1989), Ricci et al., "Differential Production of Parasiticein, and Elicitor of Necrosis and Resistance in Tobacco, by Isolates of Phytophthora parasitica," Plant Path. 41:298-307 (1992), Baillreul et al, "A New Elicitor of the Hypersensitive Response in Tobacco: A Fungal Glycoprotein Elicits Cell Death, Expression of Defence Genes, Production of Salicylic Acid, and Induction of Systemic Acquired Resistance," Plant J., 8(4):551-60 (1995), and Bonnet et al., "Acquired Resistance Triggered by Elicitors in Tobacco and Other Plants," Eur. J. Plant Path., 102:181-92 (1996), which are hereby incorporated by reference.

Another hypersensitive response elicitor in accordance with the present invention is from *Clavibacter michiganensis* subsp. s*epedonicus* which is fully described in U.S. Patent Application Serial No. 09/136,625, which is hereby incorporated by reference.

The above elicitors are exemplary. Other elicitors can be identified by growing fungi or bacteria that elicit a hypersensitive response under conditions which genes encoding an elicitor are expressed. Cell-free preparations from culture supernatants can be tested for elicitor activity (i.e. local necrosis) by using them to infiltrate appropriate plant tissues.

Fragments of the above hypersensitive response elicitor polypeptides or proteins as well as fragments of full length elicitors from other pathogens are encompassed by the method of the present invention.

Suitable fragments can be produced by several means. In the first, subclones of the gene encoding a known elicitor protein are produced by conventional molecular genetic manipulation by subcloning gene fragments. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller protein or peptide that can be tested for elicitor activity according to the procedure described below.

As an alternative, fragments of an elicitor protein can be produced by digestion of a full-length elicitor protein with proteolytic enzymes like chymotrypsin or *Staphylococcus* proteinase A, or trypsin. Different proteolytic enzymes are likely to cleave elicitor proteins at different sites based on the amino acid sequence of the elicitor protein. Some of the fragments that result from proteolysis may be active elicitors of resistance.

In another approach, based on knowledge of the primary structure of the protein, fragments of the elicitor protein gene may be synthesized by using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. These then would be cloned into an appropriate vector for expression of a truncated peptide or protein.

Chemical synthesis can also be used to make suitable fragments. Such a synthesis is carried out using known amino acid sequences for the elicitor being produced. Alternatively, subjecting a full length elicitor to high temperatures and pressures will produce fragments. These fragments can then be separated by conventional procedures (e.g., chromatography, SDS-PAGE).

An example of suitable fragments of a hypersensitive response elicitor which do elicit a hypersensitive response are *Erwinia amylovora* fragments including a C-terminal fragment of the amino acid sequence of SEQ. ID. No. 3, an N-terminal fragment of the amino acid sequence of SEQ. ID. No. 3, or an internal fragment of the amino acid sequence of SEQ. ID. No. 3. The C-terminal fragment of the amino acid sequence of SEQ. ID. No. 3 can span amino acids 105 and 403 of SEQ. ID. No. 3. The N-terminal fragment of the amino acid sequence of SEQ. ID. No. 3 can span the following amino acids of SEQ. ID. No. 3: 1 and 98, 1 and 104, 1 and 122, 1 and 168, 1 and 218, 1 and 266, 1 and 342, 1 and 321, and 1 and 372. The internal fragment of the amino acid sequence of SEQ. ID. No. 3 can span the following amino acids of

SEQ. ID. No. 3: 76 and 209, 105 and 209, 99 and 209, 137 and 204, 137 and 200, 109 and 204, 109 and 200, 137 and 180, and 105 and 180.

Suitable DNA molecules are those that hybridize to the DNA molecule comprising a nucleotide sequence of SEQ. ID. Nos. 2, 4, 5, 7, 9, 12, 13, and 16 under stringent conditions. An example of suitable high stringency conditions is when hybridization is carried out at 65°C for 20 hours in a medium containing 1M NaCl, 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.1% sodium dodecyl sulfate, 0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 50 μm g/ml *E. coli* DNA. Suitable stringency conditions also include hybridization in a hybridization buffer comprising 0.9M sodium citrate ("SSC") buffer at a temperature of 37°C where hybridized nucleic acids remain bound when subject to washing the SSC buffer at a temperature of 37°C; and preferably in a hybridization buffer comprising 20% formamide in 0.9M SSC buffer at a temperature of 42°C where hybridized nucleic acids remain bound when subject to washing at 42°C with 0.2x SSC buffer at 42°C.

Variants may be made by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

A particularly advantageous aspect of the present invention involves utilizing a protein having a pair or more, particularly 3 or more, coupled domains. These domains can be from different source organisms. When a DNA molecule encoding such a protein is prepared, it can be advantageously used to make transgenic plants. The use of a gene encoding such domains, as opposed to a gene encoding a full length hypersensitive response elicitor, has a number of benefits. Firstly, such a gene is easier to synthesize. More significantly, the use of a plurality of domains together from different source organisms can impart their combined benefits to a transgenic plant.

The DNA molecule encoding the hypersensitive response elicitor polypeptide or protein can be incorporated in cells using conventional recombinant

DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as vaccina virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F.W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria

transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promotor which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promotors differ from those of procaryotic promotors. Furthermore, eucaryotic promotors and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promotors are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

Promotors vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promotors in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promotors may be used. For instance, when cloning in *E. coli*, its

bacteriophages, or plasmids, promotors such as the T7 phage promoter, *lac* promotor, *trp* promotor, *rec*A promotor, ribosomal RNA promotor, the P_R and P_L promotors of coliphage lambda and others, including but not limited, to *lac*UV5, *omp*F, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lac*UV5 (*tac*) promotor or other *E. coli* promotors produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promotor unless specifically induced. In certain operations, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promotor, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the isolated DNA molecule encoding the hypersensitive response elicitor polypeptide or protein has been cloned into an expression system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, plant cells as well as

prokaryotic and eukaryotic cells, such as bacteria, virus, yeast, mammalian, insect cells, and the like.

The present invention further relates to methods of imparting disease resistance to plants, enhancing plant growth, effecting insect control and/or imparting stress resistance to plants. These methods involve applying a hypersensitive response elicitor polypeptide or protein to all or part of a plant or a plant seed under conditions where the polypeptide or protein contacts all or part of the cells of the plant or plant seed. Alternatively, the hypersensitive response elicitor protein or polypeptide can be applied to plants such that seeds recovered from such plants themselves are able to impart disease resistance in plants, to enhance plant growth, to effect insect control, and/or to impart stress resistance.

As an alternative to applying a hypersensitive response elicitor polypeptide or protein to plants or plant seeds in order to impart disease resistance in plants, to effect plant growth, to control insects, and/or to impart stress resistance to the plants or plants grown from the seeds, transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein and growing the plant under conditions effective to permit that DNA molecule to impart disease resistance to plants, to enhance plant growth, to control insects, and/or to impart stress resistance. Alternatively, a transgenic plant seed transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein can be provided and planted in soil. A plant is then propagated from the planted seed under conditions effective to permit that DNA molecule to impart disease resistance to plants, to enhance plant growth, to control insects, and/or to impart stress resistance.

The method of the present invention can be utilized to treat a wide variety of plants or their seeds to impart disease resistance, enhance growth, control insects, and/or to impart stress resistance. Suitable plants include dicots and monocots. More particularly, useful crop plants can include: alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, turnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash,

pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane. Examples of suitable ornamental plants are: *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

With regard to the use of the hypersensitive response elicitor protein or polypeptide of the present invention in imparting disease resistance, absolute immunity against infection may not be conferred, but the severity of the disease is reduced and symptom development is delayed. Lesion number, lesion size, and extent of sporulation of fungal pathogens are all decreased. This method of imparting disease resistance has the potential for treating previously untreatable diseases, treating diseases systemically which might not be treated separately due to cost, and avoiding the use of infectious agents or environmentally harmful materials.

The method of imparting pathogen resistance to plants in accordance with the present invention is useful in imparting resistance to a wide variety of pathogens including viruses, bacteria, and fungi. Resistance, *inter alia*, to the following viruses can be achieved by the method of the present invention: *Tobacco mosaic virus* and *Tomato mosaic virus*. Resistance, *inter alia*, to the following bacteria can also be imparted to plants in accordance with present invention: *Pseudomonas solancearum*, *Pseudomonas syringae* pv. *tabaci*, and *Xanthamonas campestris* pv. *pelargonii*. Plants can be made resistant, *inter alia*, to the following fungi by use of the method of the present invention: *Fusarium oxysporum* and *Phytophthora infestans*.

With regard to the use of the hypersensitive response elicitor protein or polypeptide of the present invention to enhance plant growth, various forms of plant growth enhancement or promotion can be achieved. This can occur as early as when plant growth begins from seeds or later in the life of a plant. For example, plant growth according to the present invention encompasses greater yield, increased quantity of seeds produced, increased percentage of seeds germinated, increased plant size, greater biomass, more and bigger fruit, earlier fruit coloration, and earlier fruit and plant maturation. As a result, the present invention provides significant economic benefit to growers. For example, early germination and early maturation permit crops to be grown in areas where short growing seasons would otherwise preclude their

growth in that locale. Increased percentage of seed germination results in improved crop stands and more efficient seed use. Greater yield, increased size, and enhanced biomass production allow greater revenue generation from a given plot of land.

Another aspect of the present invention is directed to effecting any form of insect control for plants. For example, insect control according to the present invention encompasses preventing insects from contacting plants to which the hypersensitive response elicitor has been applied, preventing direct insect damage to plants by feeding injury, causing insects to depart from such plants, killing insects proximate to such plants, interfering with insect larval feeding on such plants, preventing insects from colonizing host plants, preventing colonizing insects from releasing phytotoxins, etc. The present invention also prevents subsequent disease damage to plants resulting from insect infection.

The present invention is effective against a wide variety of insects. European corn borer is a major pest of corn (dent and sweet corn) but also feeds on over 200 plant species including green, wax, and lima beans and edible soybeans, peppers, potato, and tomato plus many weed species. Additional insect larval feeding pests which damage a wide variety of vegetable crops include the following: beet armyworm, cabbage looper, corn ear worm, fall armyworm, diamondback moth, cabbage root maggot, onion maggot, seed corn maggot, pickleworm (melonworm), pepper maggot, and tomato pinworm. Collectively, this group of insect pests represents the most economically important group of pests for vegetable production worldwide.

Another aspect of the present invention is directed to imparting stress resistance to plants. Stress encompasses any environmental factor having an adverse effect on plant physiology and development. Examples of such environmental stress include climate-related stress (e.g., drought, water, frost, cold temperature, high temperature, excessive light, and insufficient light), air polllution stress (e.g., carbon dioxide, carbon monoxide, sulfur dioxide, NO_x, hydrocarbons, ozone, ultraviolet radiation, acidic rain), chemical (e.g., insecticides, fungicides, herbicides, heavy metals), and nutritional stress (e.g., fertilizer, micronutrients, macronutrients). Use of hypersensitive response elicitors in accordance with the present invention impart resistance to plants against such forms of environmental stress.

The method of the present invention involving application of the hypersensitive response elicitor polypeptide or protein can be carried out through a variety of procedures when all or part of the plant is treated, including leaves, stems, roots, propagules (e.g., cuttings), etc. This may (but need not) involve infiltration of the hypersensitive response elicitor polypeptide or protein into the plant. Suitable application methods include high or low pressure spraying, injection, and leaf abrasion proximate to when elicitor application takes place. When treating plant seeds, in accordance with the application embodiment of the present invention, the hypersensitive response elicitor protein or polypeptide can be applied by low or high pressure spraying, coating, immersion, or injection. Other suitable application procedures can be envisioned by those skilled in the art provided they are able to effect contact of the hypersensitive response elicitor polypeptide or protein with cells of the plant or plant seed. Once treated with the hypersensitive response elicitor of the present invention, the seeds can be planted in natural or artificial soil and cultivated using conventional procedures to produce plants. After plants have been propagated from seeds treated in accordance with the present invention, the plants may be treated with one or more applications of the hypersensitive response elicitor protein or polypeptide to impart disease resistance to plants, to enhance plant growth, to control insects on the plants, and/or impart stress resistance.

The hypersensitive response elicitor polypeptide or protein can be applied to plants or plant seeds in accordance with the present invention alone or in a mixture with other materials. Alternatively, the hypersensitive response elicitor polypeptide or protein can be applied separately to plants with other materials being applied at different times.

A composition suitable for treating plants or plant seeds in accordance with the application embodiment of the present invention contains a hypersensitive response elicitor polypeptide or protein in a carrier. Suitable carriers include water, aqueous solutions, slurries, or dry powders. In this embodiment, the composition contains greater than 500 nM hypersensitive response elicitor polypeptide or protein.

Although not required, this composition may contain additional additives including fertilizer, insecticide, fungicide, nematacide, and mixtures thereof.

Suitable fertilizers include $(NH_4)_2NO_3$. An example of a suitable insecticide is Malathion. Useful fungicides include Captan.

Other suitable additives include buffering agents, wetting agents, coating agents, and abrading agents. These materials can be used to facilitate the process of the present invention. In addition, the hypersensitive response elicitor polypeptide or protein can be applied to plant seeds with other conventional seed formulation and treatment materials, including clays and polysaccharides.

In the alternative embodiment of the present invention involving the use of transgenic plants and transgenic seeds, a hypersensitive response elicitor polypeptide or protein need not be applied topically to the plants or seeds. Instead, transgenic plants transformed with a DNA molecule encoding a hypersensitive response elicitor polypeptide or protein are produced according to procedures well known in the art.

The vector described above can be microinjected directly into plant cells by use of micropipettes to transfer mechanically the recombinant DNA. Crossway, Mol. Gen. Genetics, 202:179-85 (1985), which is hereby incorporated by reference. The genetic material may also be transferred into the plant cell using polyethylene glycol. Krens, et al., Nature, 296:72-74 (1982), which is hereby incorporated by reference.

Another approach to transforming plant cells with a gene which imparts resistance to pathogens is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the heterologous DNA. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g.,

dried bacterial cells containing the vector and heterologous DNA) can also be propelled into plant cells.

Yet another method of introduction is fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible lipid-surfaced bodies. Fraley, et al., Proc. Natl. Acad. Sci. USA, 79:1859-63 (1982), which is hereby incorporated by reference.

The DNA molecule may also be introduced into the plant cells by electroporation. Fromm et al., Proc. Natl. Acad. Sci. USA, 82:5824 (1985), which is hereby incorporated by reference. In this technique, plant protoplasts are electroporated in the presence of plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

Another method of introducing the DNA molecule into plant cells is to infect a plant cell with *Agrobacterium tumefaciens* or *A. rhizogenes* previously transformed with the gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Agrobacterium is a representative genus of the gram-negative family Rhizobiaceae. Its species are responsible for crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes*). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized only by the bacteria. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In addition, assaying for the presence of opines can be used to identify transformed tissue.

Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of *A. tumefaciens* or the Ri plasmid of *A. rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells on infection by Agrobacterium and is stably integrated into the plant genome. J. Schell, Science, 237:1176-83 (1987), which is hereby incorporated by reference.

After transformation, the transformed plant cells must be regenerated.

Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co., New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. III (1986), which are hereby incorporated by reference.

It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of sugarcane, sugar beets, cotton, fruit trees, and legumes.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable.

After the expression cassette is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Once transgenic plants of this type are produced, the plants themselves can be cultivated in accordance with conventional procedure with the presence of the gene encoding the hypersensitive response elicitor resulting in disease resistance, enhanced plant growth, control of insects on the plant, and/or stress resistance. Alternatively, transgenic seeds are recovered from the transgenic plants. These seeds can then be planted in the soil and cultivated using conventional procedures to produce transgenic plants. The transgenic plants are propagated from the planted transgenic seeds under conditions effective to impart disease resistance to plants, to enhance plant growth, to control insects, and/or to impart stress resistance. While not

wishing to be bound by theory, such disease resistance, growth enhancement, insect control, and/or stress resistance may be RNA mediated or may result from expression of the elicitor polypeptide or protein.

When transgenic plants and plant seeds are used in accordance with the present invention, they additionally can be treated with the same materials as are used to treat the plants and seeds to which a hypersensitive response elicitor polypeptide or protein is applied. These other materials, including hypersensitive response elicitors, can be applied to the transgenic plants and plant seeds by the above-noted procedures, including high or low pressure spraying, injection, coating, and immersion. Similarly, after plants have been propagated from the transgenic plant seeds, the plants may be treated with one or more applications of the hypersensitive response elicitor to impart disease resistance, enhance growth, control insects, and/or to impart stress resistance. Such plants may also be treated with conventional plant treatment agents (e.g., insecticides, fertilizers, etc.).

EXAMPLES

Example 1 - Bacterial Strains and Plasmids

Escherichia coli DH5 and BL21 were purchased from Gibco BRL (Rockville, MD) and Novagen (Madison, WI) respectively.

pET28 plasmids were from Novagen (Madison, WI).

All restriction enzymes (e.g., NdeI and HindIII), T4 DNA ligase, Calf intestinal alkaline phosphatase (CIP), and PCR reagents were from Gibco BRL (Rockville, MD).

Oligonucleotides were synthesized by Lofstrand Labs Ltd (Gaithersburg, MD).

Chemically synthesized polypeptides were synthesized by Bio-Synthesis (Lewisville, TX).

Example 2 - Construction of Truncated Gene Encoding Harpin

Fragments of genes encoding harpin proteins were constructed in pET28 vector and expressed in *E. coli* as follows;

1. HrpN fragments were PCR amplified from the pCPP2139 plasmid (Cornell University, Ithaca, NY) and cloned into pET28 vector.
2. HrpZ fragments were PCR amplified from the pSYH10 plasmid (Cornell University, Ithaca, NY) and cloned into pET28 vector.
3. PopA fragments were PCR amplified from the pBS::popA plasmid (Cornell University, Ithaca, NY) and cloned into pET28 vector.
4. HrpW fragments were PCR amplified from the pCPP1233 plasmid (Cornell University, Ithaca, NY) and cloned into pET28 vector.

All truncated fragments were amplified by PCR with full length harpin DNA as the template.

Oligonucleotides corresponding to the truncated N-terminal sequence were started /modified with a Nde I site (which serves as an initiation codon of methionine (ATG)). Oligonucleotides corresponding to a C-terminal sequence contained a UAA stop codon followed by a Hind III site.

PCR was carried in a 0.5 ml tube with GeneAmpTM 9600 and 9700 (PE Applied Biosystems, Branchburg, New Jersey). 45 µl of SuperMix™ (Gibco BRL, Rockville, MD) was mixed with 20 pmoles of each pair of DNA primers, 10 ng of full length harpin DNA, and diH_2O to fill the final volume to 50 µl. After heating the mixture at 95°C for 2 min., PCR was performed for 30 cycles at 94°C for 1 min., 58°C for 1 min. and 72°C for 1.5 min. Amplified DNAs were purified with QIAquick PCR purification kit (QIAGEN Inc., Vlencia, CA), digested with Nde I and Hind III at 37°C for 5 hours, extracted once with phenol:chloroform:isoamylalcohol (25:24:1), and precipitated with ethanol. 5 µg of pET28(b) vector DNA was digested with 15 units of Nde I and 20 units of Hind III at 37°C for 3 hours followed with calf intestinal alkaline phosphatase treatment for 30 min. at 37°C to reduce the background resulting from incomplete single enzyme digestion. Digested vector DNA was purified with the QIAquick PCR purification kit and directly used for ligation. Ligation was carried at 14°C for 12 hours in a 15 µl mixture containing about 50 to

100 ng of digested pET28(b), 10 to 30 ng of targeted PCR fragments, and 1 unit of T4 DNA ligase. 5 µl of ligation solution was added to 100 µl of DH5α/XL1-Blue competent cells, placed in 15 ml Falcon tube, and incubated on ice for 30 min. After heat shock at 42°C for 45 seconds, 0.9 ml SOC solution (20 g bacto-tryptone, 5 g bacto-yeast extracts, 0.5 g NaCl, 20 mM glucose in one liter) was added into the tube and incubated at 37°C for 1 hour. 20 µl of transformed cells were plated onto LB agar plate with 30 µg/ml of kanamycin and incubated at 37°C for 14 hours. Single colonies were transferred to 3 ml LB-media and incubated overnight at 37°C. Plasmid DNA was prepared in a 2 ml culture with QIAprep Miniprep kit according to the manufacture's instruction. The DNA sequence of truncated harpin constructions was verified with restriction enzyme analysis and sequencing analysis. Plasmids with the desired DNA sequence were transferred into the BL21 strain with a standard chemical transformation method as indicated above.

Example 3 - Expression of Proteins

A single clone of *E. coli* with a constructed gene was grown overnight at 37°C in LB with kanamycin. A proper amount of overnight culture was transferred to 50 to 500 ml LB and incubated at 37°C until OD600 reached 0.5 to 0.8. ITPG was added to the culture which was further incubated at room temperature for a period of 5 hour to overnight. Alternatively, a proper amount of overnight culture was transferred to 50 to 500 ml of ½ TB with lactose medium (6 g bacto-trypton, 12 g bacto-yeast extract, 75 g lactose in one liter). After incubation at 37°C until the OD600 reached 0.5 to 0.8, the culture was incubated at room temperature for a period of 5 hours to overnight.

All bacterial cells were harvested by centrifugation and resuspended in 1:5 TE buffer (10 mM Tris, pH 8.5 and 1 mM EDTA). The cells were disrupted by sonication and clarified by centrifugation. Supernatants were then infiltrated into tobacco leaves for HR testing.

Heat treatment (i.e. boiling for 1 to 10 min.) was used to achieve further purification.

All truncated fragments of genes encoding harpin protein were expressed in *E. coli*/ BL-21, DE3 strain with an N-terminal His-tag and 20 to 21

amino acid residues generated from the expression vector sequence. The His-tag sequence did not affect the HR activity of the proteins. In some cases, Ni-Agarose beads were added into supernatant solution and mixed at 4°C to room temperature for a period of 30 min. to overnight. The proteins bound to the Ni-Agarose beads were washed by 0.1 M imidazole buffer, and proteins were eluted with 0.6 to 1.0 M imidazole. After dialysis against 10 mM Tris, pH 8.5 buffer, the proteins were infiltrated into tobacco leaves for HR testing.

For proteins expressed in *E. coli* that were difficult to dissolve in water, total cells were resuspended and sonicated in 8 M urea buffer (0.1M Na-phosphate, 10 mM Tris buffer, pH8.0). The total cell lysate was centrifuged, and supernatants were collected. Ni-agarose was added into the supernatants and mixed gently at room temperature for 30 min. The Ni-agarose resin was washed with buffer (8 M urea, 0.1 M Na-phosphate, 10 mM Tris buffer, pH6.3). The proteins were eluted with elution buffer (8 M urea, 0.1 M EDTA, 0.1 M Na-phosphate, 10 mM Tris buffer, pH 6.3) and dialyzed against buffer (pH 8.5, 10 mM Tris) with stepwise decreased urea. If the proteins still were insoluble in buffer, the solution pH was adjusted to 9 to 11 and sonicated at room temperature for 1 to 5 min.

Chemically synthesized polypeptides were dissolved in 10 mM Tris, pH 6.5 to 11 buffers depending on their solubility.

A hypersensitive response ("HR") assay was performed by infiltration of 0.1 to 0.3 ml of serial diluted protein solutions into tobacco leaves (cv. Xanth). All HR data shown in these examples were recorded from 48 hours after infiltration.

Example 4 - Quantification of Proteins

All expressed proteins were checked with pre-cast 4-20% SDS polyacrylamide gel electrophoresis (SDS-PAGE) from Novex (San Diego, CA). After electrophoresis, the gel was stained with Coomasssie R-250 solution (0.1% Coomassie R-250, 10% Acetate Acid, 40% ethanol) for 1 to 4 hours and distained with distaining solution (8% acetate acid and 25% ethanol) overnight. The density of corresponding bands were compared to standard proteins, which were either purchased from Novex or were from quantitative standard harpin protein produced by Eden Bioscience (Bothell, Washington).

Example 5 - Classification of Harpin Proteins

Since harpin proteins share common biochemical and biophysical characteristics as well as biological functions, based on their unique properties, HR elicitors from various pathogenic bacteria should be viewed as belonging to a new protein family—i.e. the harpin protein family. The harpin protein can be classified into at least four subfamilies based on their primary structure and isolated sources. As set forth in Table 1, those subfamilies are identified by the designation N, W, Z, A, etc.

Table 1 - Subfamilies of Harpin Proteins

Harpin proteins	Isolated Source	Classified Subfamily	pI	Amino acids	Heat stable	Core structure
$HrpN_{Ea}$	*E. amylovora*	N	4.42	403	Yes	No
$HrpN_{Ech}$	*E. chrysanthemi*	N	6.51	340	Yes	No
$HrpN_{Ecc}$	*E. carotovora*	N	5.82	356	Yes	No
$HrpN_{Est}$	*E. stewartii*	N	N/A	N/A	Yes	No
$HrpW_{Pss}$	*P. syringae*	W	4.43	424	Yes	No
$HrpW_{Ea}$	*E. amylovora*	W	4.46	447	Yes	No
$HrpZ_{Pss}$	*P. syringae*	Z	3.95	341	Yes	No
PopA1	*R.solanacearum*	A	4.16	344	Yes	No

Example 6 - Analysis of the Structural Units of an HR Domain

The sequence of amino acids that alone could elicit a hypersensitive response in plants (i.e. HR domains) has been investigated in different ways. It was reported that a carboxyl-terminal 148 amino acid portion of $HrpZ_{Pss}$ is sufficient and necessary for HR (He et al., "Pseudomonas Syringae pv. Syringae $Harpin_{pss}$: A Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," Cell 73:1255-1266.(1993), which is hereby incorporated by reference). With truncated HrpZ fragments, it was determined that an N-terminal 109 amino acids and C-terminal 216 amino acids of $HrpZ_{Pss}$, respectively, were found to elicit HR (Alfano et al., "Analysis of the Role of the Pseudomonas Syringae pv. Syringae HrpZ Harpin in Elicitation of the Hypersensitive Response in Tobacco Using

Functionally Non-polar hrpZ Deletion Mutations, Truncated HrpZ Fragments, and hrmA Mutations," Molecular Microbiology 19:715-728 (1996), which is hereby incorporated by reference). Jin et al., "A Truncated Fragment of $Harpin_{pss}$ Induces Systemic Resistance to Xanthomonas campestris pv. Oryzae in Rice," Physiological and Molecular Plant Pathology 51:243-257 (1997), which is hereby incorporated by reference, reported that a truncated $HrpZ_{Pss}$ with an N-terminal of 137 amino acids elicited a hypersensitive response in tobacco and induced systemic acquired resistance (i.e. SAR) in rice. After digestion with protease, a hypersensitive response active fragment of $HrpN_{Ea}$ was isolated and found to span amino acids 137 to 204 of $HrpN_{Ea}$. It was found that a 98 residue of N-terminal $HrpN_{Ea}$ fragment was the smallest bacterially produced peptide that displayed HR-eliciting activity (Laby, "Molecular Studies on Interactions Between Erwinia Amylovora and its Host and Non-host Plants," Doctoral Thesis in Cornell University (1997), which is hereby incorporated by reference).

A series of $HrpN_{Ea}$ fragments have been generated with His-tag fusion at the N-terminal of the polypeptides and a polypeptide ($HrpN_{Ea}137180$), located at position of 137 to 180 amino acid residue of $HrpN_{Ea}$, was identified to elicit HR activity in tobacco.

Example 7 - Analysis of Secondary Structure of HR Domains

The DNA and primary protein sequence of the $HrpN_{Ea}137180$ show no any homologues among other hypersensitive response elicitors.

Analyses of the secondary structure of the fragment of $HrpN_{Ea}137180$ revealed, with the aid of the computer program Clone Manger5 (Scientific & Educational Software, Durham, NC), that there was a beta-form, a beta-turn, and unordered forms. One typical α-helical segment of residues at 157-170 was found in the $HrpN_{Ea}137180$ polypeptide. To determine the function of this structure, polypeptides with a disrupted α-helical structure were generated and hypersensitive response results were evaluated. As shown in Table 2, a complete alpha-helix unit (H unit), probably with a length greater than 12 amino acid residues, is need for hypersensitive response activity.

Table 2 - Effect of Alpha-helix Structure

Fragment name	Amino acid	HR*	Structure	Source
$HrpN_{Ea}$137180	137-180 (44) pI= 3.10	+ <5 μg/ml	Complete H	E.coli expressed peptide
$HrpN_{Ea}$137166	137-166 (30) pI = 3.29	-	disrupted H	Synthesized peptide
$HrpN_{Ea}$76168	76-168 pI = 3.39	-	disrupted H	E.coli expressed peptide

The α-helical unit plays an important role in hypersensitive response activity; however, it was found that an α-helix unit alone did not achieve HR (Table 3).

Therefore, hypersensitive response eliciting domains contain more than one structure unit. Besides the core α-helical unit, there is an acidic unit that has no typical secondary structure feature but is rich in acidic amino acids. This relaxed structure, having a sheet and random turn, is designated as an acidic unit (A unit).

Although the acidic unit is important in achieving a hypersensitive response, it alone, like the α-helical unit alone, did not elicit a hypersensitive response.

A synthetic polypeptide, $HrpN_{Ea}$140176, that included both A and H structure, spanning amino acids 140 to 176 of $HrpN_{Ea}$, gave full activity of HR. Sequence analysis by major search engines revealed no global primary sequence similarity in the databases to $HrpN_{Ea}$140176, even among the harpin protein families.

Table 3 - Effect of Acidic Unit on Hypersensitive Response (HR) Activity

Fragment name	Amino acid	HR*	Structure (A or H)**	Source
$HrpN_{Ea}$140176	140-176 (37) pI=3.17	+ <5 μg/ml	A + H	Synthesized peptide
$HrpN_{Ea}$157170	157-170 (14) pI = 6.94	-	**H**	Synthesized peptide
$HrpN_{Ea}$137156	137-156 (20) pI = 2.67	-	A	Synthesized peptide

Example 8 - Hypersensitive Response Domain Structure of $HrpN_{Ea}$

Four α-helical regions with at least 12 amino acid residues were found in $HrpN_{Ea}$ based on computer analysis with the program Clone Manager 5 (Scientific & Educational Software, Durham, NC), which predicts the secondary structure of protein from the primary sequence by the method of Garnier-Osguthorpe-Robson.

It is believed that a hypersensitive response domain includes two structural units, the α-helix (H) and the acidic unit (A). Another hypersensitive response domain, spanning amino acids 43 to 70 in HrpNEa, was found. A minimal sequence of 12 to 14 AA residues of both the H and A units is believed to be needed. The chemically synthesized polypeptide of $HrpN_{Ea}4370$ gave full HR activity in tobacco. Thus, a second HR domain has been discovered based on purely secondary structure analysis and prediction.

To further test the hypothesis that the A and H units are needed to achieve a hypersensitive response, an approach of unit exchange (i.e. swapping an acidic unit from one HR domain to another HR domain) was designed. A polypeptide of $HrpN_{Ea}$Dswap, which consisted of the acidic unit of a hypersensitive response domain ($HrpN_{Ea}140176$), spanning amino acids 136 to 156 of $HrpN_{Ea}$, and the α-helical unit of another hypersensitive response domain ($HrpN_{Ea}4370$), spanning amino acids 57 to 70 of $HrpN_{Ea}$, was chemically synthesized. This polypeptide swapped two structural units of A and H between two hypersensitive response domains of $HrpN_{Ea}4370$ and $HrpN_{Ea}140176$. The $HrpN_{Ea}$Dswap gave a hypersensitive response activity in tobacco (Table 4). This result shows that the structural characteristic of an HR domain determines its activity, and structural analysis can be used to determine hypersensitive response activity.

Table 4 - Two Structural Units Determine Hypersensitive Response Activity

Fragment name	Amino acid	HR	Structure Type	Source
$HrpN_{Ea}4370$	43-70 (28) pI= 3.09	+ <5 μg/ml	A + H	Synthesized peptide Partial soluble
$HrpN_{Ea}$Dswap	HrpN136156 (A)+ HrpN5770 (H) pI=2.67	<20 μg/ml	A unit from $HrpN_{Ea}140176$ + H unit from $HrpN_{Ea}4370$	Synthesized peptide Partial soluble

Example 9 - Prediction of Hypersensitive Response Domains Among Proteins in Harpin Family

The secondary structure which indicates the presence of a hypersensitive response domain in HrpNEa was used to identify other harpin proteins, including proteins classified as different subfamilies. Structural prediction of a hypersensitive response domain among harpin proteins was carried according to following criteria:

1. There are two structural units in a hypersensitive response domain, including:
   a. A stable α-helix unit with 12 or more amino acids in length and
   b. An hydrophilic, acidic unit with 12 or more amino acids in length which could be a beta-form, a beta-turn, and unordered forms.
2. The pI of a hypersensitive response domain should be acidic and, in general, below 5.
3. The minimal size of an HR domain is from about 28 to 40 AA residues.

Putative HR domains have been identified to fit the criteria by computer analysis among harpin protein family (Table 5).

Table 5 - Predication of Hypersensitive Response Domains Among Harpin Proteins

HR domain	Isolated Source	Predicted region*	pI	Structure
$HrpN_{Ea}$-1	*E. amylovora*	43-70	3.09	A + H
$HrpN_{Ea}$-2	*E. amylovora*	140-176	3.17	A + H
$HrpN_{Ech}$-1	*E. chrysanthemi*	78-118	5.25	A + H
$HrpN_{Ech}$-2	*E. chrysanthemi*	256-295	4.62	A + H
$HrpN_{Ecc}$-1	*E. carotovora*	25-63	4.06	A + H
$HrpN_{Ecc}$-2	*E. carotovora*	101-140	3.00	A + H
$HrpW_{Pss}$-1	*P. syringae*	52-96	4.32	A + H
$HrpW_{Ea}$-1	*E. amylovora*	10-59	4.53	A + H
$HrpZ_{Pss}$-1	*P. syringae*	97-132	3.68	A + H
$HrpZ_{Pss}$-2	*P. syringae*	153-189	3.67	A + H
$HrpZ_{Pss}$-3	*P. syringae*	271-308	3.95	A + H
$PopA1_{Rs}$-1	*R.solanacearum*	92-125	3.75	A + H
$PopA1_{Rs}$-2	*R.solanacearum*	206-260	3.62	A + H

*Amino acid residue position

Example 10 - Hypersensitive Response Activity of Select Synthesized Polypeptides

Polypeptides were produced by expression in either *E. coli* or by chemical synthesis. Based on prediction of solubility and stability of a particular peptide, in some cases, a broader region of AA residues in addition to the essential units were also synthesized to increase solubility of the peptides. The identification of HR domains among four subfamilies of harpin protein demonstrated this (Table 6).

Table 6 - Hypersensitive Response Activity of Select Synthesized Polypeptides

HR domain	Isolated Source	Synthesized region	pI	Source	HR activity
$HrpN_{Ea}$-1	*E. amylovora*	43-70	3.09	Chemical Synthesized	+ < 5 μg/ml
$HrpN_{Ea}$-2	*E. amylovora*	140-176	3.17	Chemical Synthesized	+ < 5 μg/ml
$HrpW_{Ea}$-2	*E. amylovora*	10-59	4.53	E.coli expressed	+ < 5 μg/ml
$HrpZ_{Pss}$-1	*P. syringae*	97-132	3.68	Chemical Synthesized	+ < 20 μg/ml
$HrpZ_{Pss}$-1	*P. syringae*	153-189	3.69	E.coli expressed	+ < 5 μg/ml
$PopA1_{Rs}$-1	*R. solanacearum*	92-125	3.75	Chemical Synthesized	+ < 5 μg/ml
$PopA1_{Rs}$-2	*R. solanacearum*	206-260	3.62	E.coli expressed	+ < 5 μg/ml

Example 11 - Construction of Hypersensitive Response Domains in a Protein Expression Cassette

Polypeptides with a harpin protein hypersensitive response domain were expressed in *E. coli*. PCR was used to amplify desired areas of genes encoding harpin proteins and cloned into an expression vector, e.g. pET28a. A pair of PCR primers with unique flanking sequences were designed to create a universal expression cassette, as shown in Figure 1, for expression of a fragment of harpin protein. Each amplified DNA fragment has a protein translation start codon of ATG in a restriction enzyme Nde I site which might add an extra amino acid of methionine into a polypeptide. Each amplified DNA fragment has a protein translation stop codon of TAA. Each amplified fragment contained two restriction enzyme sites of EcoR V and Sma I, which gave 4 extra in-frame amino acids expressed as Pro-Gly at the N-terminal and Asp-Ile at the C-terminal, respectively. Those two sites are essential to allow two or more expression cassettes to be linked in a specific order and in frame with a minimum number of amino acids being introduced. Cassette A was first digested by EcoR V, ligated to cassette B, and digested with Sma I to produce a new expression cassette C which coupled the two fragments together with two extra amino acids (i.e. Asp-Gly), which are common amino acids in hypersensitive response domains. The newly formed cassette C still contained the same 5' and 3' flanking sequences as original cassettes A and B and maintained the ability to be

coupled by another cassette. Bgl II and Bam HI sites in the cassette permit the cassette to be linked in frame into a cancatomer with a correct orientation. The strategy is that digestion of DNA with Bgl II and Bam HI results in compatible ends that would be ligated with each other but could not be cut by either enzymes after ligation. For example, a DNA fragment encoding a hypersensitive response domain in a cassette could be digested by restrictions enzymes of Bgl II and Bam H1 separately, digested DNA fragments could be ligated in a ligation solution also including both Bgl II and Bam HI enzymes, any ligated ends with Bgl II or Bam HI sites could be digested by the enzymes, and only those ligated sites between Bgl II and Bam HI could remain.

Example 12 - Building Blocks for Creating Superharpins that have Higher Biological Efficacy

Hypersensitive response domains were identified and isolated from several harpin proteins. With the combination of those HR domains, new polypeptides (i.e. superharpins) that have higher HR potency and have enhanced ability to induce disease resistance, impart insect resistance, enhance growth, and achieve environmental stress tolerance. Superharpins could be one HR domain repeat units (cancatomer), different combinations of HR domains, and/or biologically active domains from other elicitors. Part of the domains from different harpin proteins and other elicitors were constructed into the universal expression cassette as shown on Example 11 and designated as superharpin building blocks. Table 7 lists some superharpin building blocks which were expressed in pET-28a(+) vector with a His-tag sequence at their N-terminal.

Table 7 - Superharpin Building Blocks including pET-28a(+) his-tag Leader Sequence

Domain Sequence	Source	MW (kDa)	#a.a.	pI	Soluble	(Structurally) Heat Stable
A	PopA70-146	10.69	104	6.48	Yes	Yes
(N_N)	HrpNEa40-80	6.754	68	6.78	N/A	N/A
$(N_N)_2$	Dimer of HrpNEa40-80	10.84	111	6.13	N/A	N/A
$(N_N)_3$	Triplemer of HrpNEa40-80	14.93	154	5.63	N/A	N/A
$(N_N)_4$	Tetramer of HrpNEa40-80	19.01	197	4.95	N/A	N/A
(N_C)	HrpNEa140-180	7.224	68	5.01	Yes	Yes
$(N_C)_2$	Dimer of HrpNEa140-180	11.78	111	3.98	Yes	Yes
$(N_C)_3$	Triplemer of HrpNEa140-180	16.34	154	3.72	Yes	Yes
$(N_C)_4$	Tetramer of HrpNEa140-180	20.89	197	3.58	Yes	Yes
$(N_C)_{10}$	Cancatomer (10 repeating units of HrpNEa140-180	48.23	455	3.28	N/A	N/A
$(N_C)_{16}$	Cancatomer (16 repeating units of HrpNEa140-180	75.57	713	3.18	N/A	N/A
W	HrpWEa10-59	7.986	77	6.48	N/A	N/A
Z_N	HrpZ90-150	8.087	78	5.38	Yes	Yes
$Z_{266\text{-}308}$	HrpZ266-308	7.029	70	6.40	Yes	Yes
his-tag leader seq.		2.045	19	11.04		

Example 13 - Superharpins with Stacked HR Domains and their Biological Activities

There are numerous polypeptides could be generated with different combinations of HR domains or by stacking HR domains and repeating units in order. Selective combination or stacking of HR domains isolated from harpin proteins or other elicitors can be designed to achieve a targeted disease resistance spectrum. See Table 8 for superharpins prepared by stacking of HR building blocks listed on Table 7. All three listed superharpins (i.e. SH-1, SH-2, SH-3) were constructed into a pET28(a) vector and expressed in *E. coli*. Recombinant proteins were partially purified and quantified by SDS-PAGE with purified Harpin N protein as a quantitative standard.

Table 8 - Properties of Superharpins

Protein	Domain Sequence	MW (kDa)	# a.a.	pI	Soluble	Heat Stable
SH-1	*$W(N_N)_4A(N_C)_4Z_{266\text{-}308}$	54.955	545	3.69	Yes	Yes
SH-2	*$W(N_N)_4Z_N(N_C)_4Z_{266\text{-}308}$	52.341	519	3.54	Yes	Yes
SH-3	*$W(N_N)_4Z_N(N_C)_4Z_{266\text{-}308}A$	60.375	598	3.67	Yes	Yes
HrpNEa	HrpN from E.amylovora	39.697	403	4.42	Yes	Yes

Bioassays for hypersensitive response on tobacco leaves (HR), percentage of TMV reduction on tobacco leaves, and plant growth enhancement with tomato showed that superharpins had higher (up to 2 to 10 fold greater) HR potency compared with HrpN from *E. amylovora*. This also demonstrated that superharpins have better performance on % TMV reduction and plant growth enhancement assay. See Table 9.

Table 9 - Biological Activities of Superharpins

Protein	Domain Sequence	Elicit HR (~µg/ml)	% TMV reduction on tobacco		% Plant Growth Enhancement	
			10 µg/ml	1 µg/ml	10 µg/ml	1 µg/ml
SH-1	$W(N_N)_4A(N_C)_4Z_{266\text{-}308}$	0.66	83	79	7.49	9.83
SH-2	$W(N_N)_4Z_N(N_C)_4Z_{266\text{-}308}$	0.13	84	60	11.05	7.30
SH-3	$W(N_N)_4Z_N(N_C)_4Z_{266\text{-}308}A$	0.15	77	55	11.07	10.00
HrpNEa	HrpN from E.amylovora	1-3	55	10	11.68	N/A

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

WHAT IS CLAIMED:

1. An isolated hypersensitive response elicitor protein comprising an isolated pair or more of spaced apart domains, each comprising an acidic portion linked to an alpha-helix and capable of eliciting a hypersensitive response in plants.

2. A protein according to claim 1, wherein the protein is recombinant.

3. An isolated nucleic acid molecule encoding a protein according to claim 1.

4. A nucleic acid molecule according to claim 3, wherein each domain is from a different source organism.

5. A nucleic acid molecule according to claim 3, wherein there are 3 or more spaced apart domains.

6. An expression vector containing a nucleic acid molecule according to claim 3 which is heterologous to the expression vector.

7. An expression vector according to claim 6, wherein the nucleic acid molecule is positioned in the expression vector in sense orientation and correct reading frame.

8. A host cell transformed with the nucleic acid molecule according to claim 3.

9. A host cell transformed according to claim 8, wherein the host cell is selected from the group consisting of a plant cell, a eukaryotic cell, and a procaryotic cell.

10. A host cell according to claim 8, wherein the nucleic acid molecule is transformed with an expression system.

11. A transgenic plant transformed with the nucleic acid molecule of claim 3.

12. A transgenic plant according to claim 11, wherein the plant is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

13. A transgenic plant according to claim 11, wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

14. A transgenic plant according to claim 11, wherein the plant is a monocot.

15. A transgenic plant according to claim 11, wherein the plant is a dicot.

16. A transgenic plant according to claim 11, wherein each domain is from a different source organism.

17. A transgenic plant according to claim 11, wherein there are 3 or more spaced apart domains.

18. A transgenic plant seed transformed with the nucleic acid molecule of claim 3.

19. A transgenic plant seed according to claim 18, wherein the plant is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

20. A transgenic plant seed according to claim 18, wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

21. A transgenic plant seed according to claim 18, wherein the plant is a monocot.

22. A transgenic plant seed according to claim 18, wherein the plant is a dicot.

23. A method of imparting disease resistance to plants comprising:

applying a protein according to claim 1 to a plant or a plant seed under conditions effective to impart disease resistance to the plant or to a plant grown from the plant seed.

24. A method according to claim 23, wherein the protein is applied to a plant.

25. A method according to claim 23, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to impart disease resistance to a plant grown from the plant seeds.

26. A method of enhancing plant growth comprising:

applying a protein according to claim 1 to a plant or a plant seed under conditions effective to enhance growth of the plants or of a plant grown from the plant seed.

27. A method according to claim 26, wherein the protein is applied to a plant.

28. A method according to claim 26, wherein the protein is applied to a plant seed and further comprising:

planting the plant seeds under conditions effective to enhance growth of a plant grown from the plant seed.

29. A method of controlling insects comprising:

applying a protein according to claim 1 to a plant or a plant seed under conditions effective to control insects.

30. A method according to claim 29, wherein the protein is applied to a plant.

31. A method according to claim 29, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to grow a plant from the plant seed and to control insects.

32. A method of imparting stress resistance to plants comprising:

applying a protein according to claim 1 to a plant or a plant seed under conditions effective to impart stress resistance to the plant or to a plant grown from the plant seed.

33. A method according to claim 32, wherein the protein is applied to a plant.

34. A method according to claim 32, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to impart stress resistance to a plant grown from the plant seed.

35. A method of imparting disease resistance to plants comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 3 and

planting the transgenic plant or transgenic plant seed under conditions effective to impart disease resistance to the plant or to a plant grown from the plant seed.

36. A method according to claim 35, wherein a transgenic plant is provided.

37. A method according to claim 35, wherein a transgenic plant seed is provided.

38. A method of enhancing growth of plants comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 3 and

planting the transgenic plant or transgenic plant seed under conditions effective to enhance growth of the plant or of a plant grown from the plant seed.

39. A method according to claim 38, wherein a transgenic plant is provided.

40. A method according to claim 38, wherein a transgenic plant seed is provided.

41. A method of controlling insects comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 3 and

planting the transgenic plant or transgenic plant seed under conditions effective to control insects on the plant or on a plant grown from the plant seed.

42. A method according to claim 41, wherein a transgenic plant is provided.

43. A method according to claim 41, wherein a transgenic plant seed is provided.

44. A method of imparting stress resistance to plants comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 3 and

planting the transgenic plant or transgenic plant seed under conditions effective to impart stress resistance to the plant or to a plant grown from the plant seed.

45. A method according to claim 44, wherein a transgenic plant is provided.

46. A method according to claim 44, wherein a transgenic plant seed is provided.

47. An isolated hypersensitive response elicitor protein comprising, in isolation, a domain comprising an acid portion linked to an alpha-helix and capable of eliciting a hypersensitive response in plants.

48. A protein according to claim 47, wherein the protein is recombinant.

49. An isolated nucleic acid molecule encoding a protein according to claim 47.

50. An isolated nucleic acid molecule according to claim 49, wherein there are at least 2 domains, each from a different source organism.

51. An isolated nucleic acid molecule according to claim 49, wherein there are 3 or more coupled domains.

52. An expression vector containing a nucleic acid molecule according to claim 49 which is heterologous to the expression vector.

53. An expression vector according to claim 52, wherein the nucleic acid molecule is positioned in the expression vector in sense orientation and correct reading frame.

54. A host cell transformed with the nucleic acid molecule according to claim 49.

55. A host cell transformed according to claim 54, wherein the host cell is selected from the group consisting of a plant cell, a eukaryotic cell, and a prokaryotic cell.

56. A host cell according to claim 54, wherein the nucleic acid molecule is transformed with an expression system.

57. A transgenic plant transformed with the nucleic acid molecule of claim 49.

58. A transgenic plant according to claim 57, wherein the plant is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean pea, chicory, lettuce, endive,

cabbage, brussel sprout, beet, parsnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

59. A transgenic plant according to claim 57, wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

60. A transgenic plant according to claim 57, wherein the plant is a monocot.

61. A transgenic plant according to claim 57, wherein the plant is a dicot.

62. A transgenic plant according to claim 57, wherein there are at least 2 coupled domains, each from a different source organism.

63. A transgenic plant according to claim 57, wherein there are 3 or more coupled domains.

64. A transgenic plant seed transformed with the nucleic acid molecule of claim 49.

65. A transgenic plant seed according to claim 64, wherein the plant is selected from the group consisting of alfalfa, rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean pea, chicory, lettuce, endive, cabbage, brussel sprout, beet, parsnip, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, citrus, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

66. A transgenic plant seed according to claim 64, wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, *Saintpaulia*, petunia, pelargonium, poinsettia, chrysanthemum, carnation, and zinnia.

67. A transgenic plant seed according to claim 64, wherein the plant is a monocot.

68. A transgenic plant seed according to claim 64, wherein the plant is a dicot.

69. A method of imparting disease resistance to plants comprising:

applying a protein according to claim 47 to a plant or a plant seed under conditions effective to impart disease resistance to the plant or to a plant grown from the plant seed.

70. A method according to claim 69, wherein the protein is applied to a plant.

71. A method according to claim 69, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to impart disease resistance to a plant grown from the plant seed.

72. A method of enhancing plant growth comprising:

applying a protein according to claim 47 to a plant or a plant seed under conditions effective to enhance growth of the plant or of a plant grown from the plant seed.

73. A method according to claim 72, wherein the protein is applied to a plant.

74. A method according to claim 72, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to enhance growth of a plant grown from the plant seed.

75. A method of controlling insects comprising:

applying a protein according to claim 47 to a plant or a plant seed under conditions effective to control insects.

76. A method according to claim 75, wherein the protein is applied to a plant.

77. A method according to claim 75, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to grow a plant from the plant seed and to control insects.

78. A method of imparting stress resistance to plants comprising:

applying a protein according to claim 47 to a plant or a plant seed under conditions effective to impart stress resistance to the plant or to a plant grown from the plant seed.

79. A method according to claim 78, wherein the protein is applied to a plant.

80. A method according to claim 78, wherein the protein is applied to a plant seed and further comprising:

planting the plant seed under conditions effective to impart stress resistance to a plant grown from the plant seed.

81. A method of imparting disease resistance to plants comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 49 and

planting the transgenic plant or transgenic plant seed under conditions effective to impart disease resistance to the plant or to a plant grown from the plant seed.

82. A method according to claim 81, wherein a transgenic plant is provided.

83. A method according to claim 81, wherein a transgenic plant seed is provided.

84. A method of enhancing growth of plants comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 49 and

planting the transgenic plant or transgenic plant seed under conditions effective to enhance growth of the plant or of a plant grown from the plant seed.

85. A method according to claim 84, wherein a transgenic plant is provided.

86. A method according to claim 84, wherein a transgenic plant seed is provided.

87. A method of controlling insects comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 49 and

planting the transgenic plant or transgenic plant seed under conditions effective to control insects on the plant or on a plant grown from the plant seed.

88. A method according to claim 87, wherein a transgenic plant is provided.

89. A method according to claim 87, wherein a transgenic plant seed is provided.

90. A method of imparting stress resistance to plants comprising:

providing a transgenic plant or transgenic plant seed containing the nucleic acid according to claim 49 and

planting the transgenic plant or transgenic plant seed under conditions effective to impart stress resistance to the plant or to a plant grown from the plant seed.

91. A method according to claim 90, wherein a transgenic plant is provided.

92. A method according to claim 90, wherein a transgenic plant seed is provided.

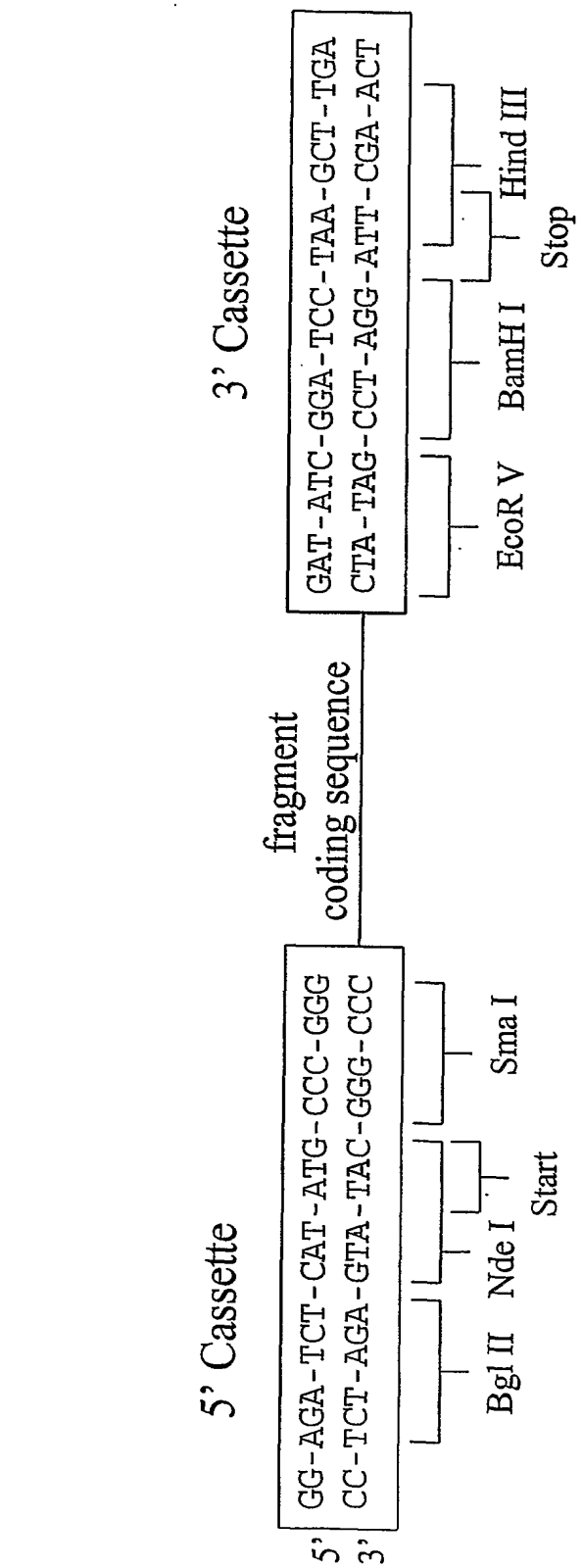
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Figure 1

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<213> Erwinia amylovora

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<211> 447
<212> PRT
<213> Erwinia amylovora

<400> 6

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Phe Gln Ser Gly Gly Asp Asn Gly Leu Gly Gly His Asn Ala Asn Ser
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Ala Leu Gly Gln Gln Pro Ile Asp Arg Gln Thr Ile Glu Gln Met Ala
        35                  40                  45

Gln Leu Leu Ala Glu Leu Leu Lys Ser Leu Leu Ser Pro Gln Ser Gly
    50                  55                  60

Asn Ala Ala Thr Gly Ala Gly Gly Asn Asp Gln Thr Thr Gly Val Gly
65                  70                  75                  80

Asn Ala Gly Gly Leu Asn Gly Arg Lys Gly Thr Ala Gly Thr Thr Pro
                85                  90                  95

Gln Ser Asp Ser Gln Asn Met Leu Ser Glu Met Gly Asn Asn Gly Leu
            100                 105                 110

Asp Gln Ala Ile Thr Pro Asp Gly Gln Gly Gly Gly Gln Ile Gly Asp
        115                 120                 125

Asn Pro Leu Leu Lys Ala Met Leu Lys Leu Ile Ala Arg Met Met Asp
    130                 135                 140

Gly Gln Ser Asp Gln Phe Gly Gln Pro Gly Thr Gly Asn Asn Ser Ala
145                 150                 155                 160

Ser Ser Gly Thr Ser Ser Ser Gly Gly Ser Pro Phe Asn Asp Leu Ser
                165                 170                 175

Gly Gly Lys Ala Pro Ser Gly Asn Ser Pro Ser Gly Asn Tyr Ser Pro
            180                 185                 190

Val Ser Thr Phe Ser Pro Pro Ser Thr Pro Thr Ser Pro Thr Ser Pro
        195                 200                 205

Leu Asp Phe Pro Ser Ser Pro Thr Lys Ala Ala Gly Gly Ser Thr Pro
    210                 215                 220

Val Thr Asp His Pro Asp Pro Val Gly Ser Ala Gly Ile Gly Ala Gly
225                 230                 235                 240

Asn Ser Val Ala Phe Thr Ser Ala Gly Ala Asn Gln Thr Val Leu His
                245                 250                 255
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Asp Thr Ile Thr Val Lys Ala Gly Gln Val Phe Asp Gly Lys Gly Gln
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Thr Phe Thr Ala Gly Ser Glu Leu Gly Asp Gly Gly Gln Ser Glu Asn
        275                 280                 285

Gln Lys Pro Leu Phe Ile Leu Glu Asp Gly Ala Ser Leu Lys Asn Val
    290                 295                 300

Thr Met Gly Asp Asp Gly Ala Asp Gly Ile His Leu Tyr Gly Asp Ala
305                 310                 315                 320

Lys Ile Asp Asn Leu His Val Thr Asn Val Gly Glu Asp Ala Ile Thr
                325                 330                 335

Val Lys Pro Asn Ser Ala Gly Lys Lys Ser His Val Glu Ile Thr Asn
            340                 345                 350

Ser Ser Phe Glu His Ala Ser Asp Lys Ile Leu Gln Leu Asn Ala Asp
        355                 360                 365

Thr Asn Leu Ser Val Asp Asn Val Lys Ala Lys Asp Phe Gly Thr Phe
    370                 375                 380

Val Arg Thr Asn Gly Gly Gln Gln Gly Asn Trp Asp Leu Asn Leu Ser
385                 390                 395                 400

His Ile Ser Ala Glu Asp Gly Lys Phe Ser Phe Val Lys Ser Asp Ser
                405                 410                 415

Glu Gly Leu Asn Val Asn Thr Ser Asp Ile Ser Leu Gly Asp Val Glu
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Asn His Tyr Lys Val Pro Met Ser Ala Asn Leu Lys Val Ala Glu
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<211> 5517
<212> DNA
<213> Erwinia amylovora

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<210> 8
<211> 1838
<212> PRT
<213> Erwinia amylovora

<400> 8
Met Glu Leu Lys Ser Leu Gly Thr Glu His Lys Ala Ala Val His Thr

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            20                  25                  30

Ser Ser Ser Ser Pro Gln Asn Ala Ala Ala Ser Leu Ala Ala Glu Gly
        35                  40                  45

Lys Asn Arg Gly Lys Met Pro Arg Ile His Gln Pro Ser Thr Ala Ala
    50                  55                  60

Asp Gly Ile Ser Ala Ala His Gln Gln Lys Lys Ser Phe Ser Leu Arg
65                  70                  75                  80

Gly Cys Leu Gly Thr Lys Lys Phe Ser Arg Ser Ala Pro Gln Gly Gln
                85                  90                  95

Pro Gly Thr Thr His Ser Lys Gly Ala Thr Leu Arg Asp Leu Leu Ala
            100                 105                 110

Arg Asp Asp Gly Glu Thr Gln His Glu Ala Ala Ala Pro Asp Ala Ala
        115                 120                 125

Arg Leu Thr Arg Ser Gly Gly Val Lys Arg Arg Asn Met Asp Asp Met
    130                 135                 140

Ala Gly Arg Pro Met Val Lys Gly Gly Ser Gly Glu Asp Lys Val Pro
145                 150                 155                 160

Thr Gln Gln Lys Arg His Gln Leu Asn Asn Phe Gly Gln Met Arg Gln
                165                 170                 175

Thr Met Leu Ser Lys Met Ala His Pro Ala Ser Ala Asn Ala Gly Asp
            180                 185                 190

Arg Leu Gln His Ser Pro Pro His Ile Pro Gly Ser His His Glu Ile
        195                 200                 205

Lys Glu Glu Pro Val Gly Ser Thr Ser Lys Ala Thr Thr Ala His Ala
    210                 215                 220

Asp Arg Val Glu Ile Ala Gln Glu Asp Asp Asp Ser Glu Phe Gln Gln
225                 230                 235                 240

Leu His Gln Gln Arg Leu Ala Arg Glu Arg Glu Asn Pro Pro Gln Pro
                245                 250                 255

Pro Lys Leu Gly Val Ala Thr Pro Ile Ser Ala Arg Phe Gln Pro Lys
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Leu Thr Ala Val Ala Glu Ser Val Leu Glu Gly Thr Asp Thr Thr Gln
        275                 280                 285

Ser Pro Leu Lys Pro Gln Ser Met Leu Lys Gly Ser Gly Ala Gly Val
    290                 295                 300

Thr Pro Leu Ala Val Thr Leu Asp Lys Gly Lys Leu Gln Leu Ala Pro
305                 310                 315                 320

Asp Asn Pro Pro Ala Leu Asn Thr Leu Leu Lys Gln Thr Leu Gly Lys
                325                 330                 335

Asp Thr Gln His Tyr Leu Ala His His Ala Ser Ser Asp Gly Ser Gln
            340                 345                 350

His Leu Leu Leu Asp Asn Lys Gly His Leu Phe Asp Ile Lys Ser Thr
        355                 360                 365

Ala Thr Ser Tyr Ser Val Leu His Asn Ser His Pro Gly Glu Ile Lys
    370                 375                 380

Gly Lys Leu Ala Gln Ala Gly Thr Gly Ser Val Ser Val Asp Gly Lys
385                 390                 395                 400

Ser Gly Lys Ile Ser Leu Gly Ser Gly Thr Gln Ser His Asn Lys Thr
                405                 410                 415

Met Leu Ser Gln Pro Gly Glu Ala His Arg Ser Leu Leu Thr Gly Ile
            420                 425                 430

Trp Gln His Pro Ala Gly Ala Ala Arg Pro Gln Gly Glu Ser Ile Arg
        435                 440                 445

Leu His Asp Asp Lys Ile His Ile Leu His Pro Glu Leu Gly Val Trp
    450                 455                 460

Gln Ser Ala Asp Lys Asp Thr His Ser Gln Leu Ser Arg Gln Ala Asp
465                 470                 475                 480

Gly Lys Leu Tyr Ala Leu Lys Asp Asn Arg Thr Leu Gln Asn Leu Ser
                485                 490                 495

Asp Asn Lys Ser Ser Glu Lys Leu Val Asp Lys Ile Lys Ser Tyr Ser
            500                 505                 510

Val Asp Gln Arg Gly Gln Val Ala Ile Leu Thr Asp Thr Pro Gly Arg
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        515                 520                 525

His Lys Met Ser Ile Met Pro Ser Leu Asp Ala Ser Pro Glu Ser His
    530                 535                 540

Ile Ser Leu Ser Leu His Phe Ala Asp Ala His Gln Gly Leu Leu His
545                 550                 555                 560

Gly Lys Ser Glu Leu Glu Ala Gln Ser Val Ala Ile Ser His Gly Arg
                565                 570                 575

Leu Val Val Ala Asp Ser Glu Gly Lys Leu Phe Ser Ala Ala Ile Pro
            580                 585                 590

Lys Gln Gly Asp Gly Asn Glu Leu Lys Met Lys Ala Met Pro Gln His
        595                 600                 605

Ala Leu Asp Glu His Phe Gly His Asp His Gln Ile Ser Gly Phe Phe
    610                 615                 620

His Asp Asp His Gly Gln Leu Asn Ala Leu Val Lys Asn Asn Phe Arg
625                 630                 635                 640

Gln Gln His Ala Cys Pro Leu Gly Asn Asp His Gln Phe His Pro Gly
                645                 650                 655

Trp Asn Leu Thr Asp Ala Leu Val Ile Asp Asn Gln Leu Gly Leu His
            660                 665                 670

His Thr Asn Pro Glu Pro His Glu Ile Leu Asp Met Gly His Leu Gly
        675                 680                 685

Ser Leu Ala Leu Gln Glu Gly Lys Leu His Tyr Phe Asp Gln Leu Thr
    690                 695                 700

Lys Gly Trp Thr Gly Ala Glu Ser Asp Cys Lys Gln Leu Lys Lys Gly
705                 710                 715                 720

Leu Asp Gly Ala Ala Tyr Leu Leu Lys Asp Gly Glu Val Lys Arg Leu
                725                 730                 735

Asn Ile Asn Gln Ser Thr Ser Ser Ile Lys His Gly Thr Glu Asn Val
            740                 745                 750

Phe Ser Leu Pro His Val Arg Asn Lys Pro Glu Pro Gly Asp Ala Leu
        755                 760                 765

Gln Gly Leu Asn Lys Asp Asp Lys Ala Gln Ala Met Ala Val Ile Gly
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    770                 775                 780

Val Asn Lys Tyr Leu Ala Leu Thr Glu Lys Gly Asp Ile Arg Ser Phe
785                 790                 795                 800

Gln Ile Lys Pro Gly Thr Gln Gln Leu Glu Arg Pro Ala Gln Thr Leu
                805                 810                 815

Ser Arg Glu Gly Ile Ser Gly Glu Leu Lys Asp Ile His Val Asp His
            820                 825                 830

Lys Gln Asn Leu Tyr Ala Leu Thr His Glu Gly Glu Val Phe His Gln
        835                 840                 845

Pro Arg Glu Ala Trp Gln Asn Gly Ala Glu Ser Ser Ser Trp His Lys
    850                 855                 860

Leu Ala Leu Pro Gln Ser Glu Ser Lys Leu Lys Ser Leu Asp Met Ser
865                 870                 875                 880

His Glu His Lys Pro Ile Ala Thr Phe Glu Asp Gly Ser Gln His Gln
                885                 890                 895

Leu Lys Ala Gly Gly Trp His Ala Tyr Ala Ala Pro Glu Arg Gly Pro
            900                 905                 910

Leu Ala Val Gly Thr Ser Gly Ser Gln Thr Val Phe Asn Arg Leu Met
        915                 920                 925

Gln Gly Val Lys Gly Lys Val Ile Pro Gly Ser Gly Leu Thr Val Lys
    930                 935                 940

Leu Ser Ala Gln Thr Gly Gly Met Thr Gly Ala Glu Gly Arg Lys Val
945                 950                 955                 960

Ser Ser Lys Phe Ser Glu Arg Ile Arg Ala Tyr Ala Phe Asn Pro Thr
                965                 970                 975

Met Ser Thr Pro Arg Pro Ile Lys Asn Ala Ala Tyr Ala Thr Gln His
            980                 985                 990

Gly Trp Gln Gly Arg Glu Gly Leu Lys Pro Leu Tyr Glu Met Gln Gly
        995                1000                1005

Ala Leu Ile Lys Gln Leu Asp Ala His Asn Val Arg His Asn Ala Pro
   1010                1015                1020

Gln Pro Asp Leu Gln Ser Lys Leu Glu Thr Leu Asp Leu Gly Glu His
```

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Gly Ala Glu Leu Leu Asn Asp Met Lys Arg Phe Arg Asp Glu Leu Glu
                1045                1050                1055

Gln Ser Ala Thr Arg Ser Val Thr Val Leu Gly Gln His Gln Gly Val
            1060                1065                1070

Leu Lys Ser Asn Gly Glu Ile Asn Ser Glu Phe Lys Pro Ser Pro Gly
        1075                1080                1085

Lys Ala Leu Val Gln Ser Phe Asn Val Asn Arg Ser Gly Gln Asp Leu
    1090                1095                1100

Ser Lys Ser Leu Gln Gln Ala Val His Ala Thr Pro Pro Ser Ala Glu
1105                1110                1115                1120

Ser Lys Leu Gln Ser Met Leu Gly His Phe Val Ser Ala Gly Val Asp
                1125                1130                1135

Met Ser His Gln Lys Gly Glu Ile Pro Leu Gly Arg Gln Arg Asp Pro
            1140                1145                1150

Asn Asp Lys Thr Ala Leu Thr Lys Ser Arg Leu Ile Leu Asp Thr Val
        1155                1160                1165

Thr Ile Gly Glu Leu His Glu Leu Ala Asp Lys Ala Lys Leu Val Ser
    1170                1175                1180

Asp His Lys Pro Asp Ala Asp Gln Ile Lys Gln Leu Arg Gln Gln Phe
1185                1190                1195                1200

Asp Thr Leu Arg Glu Lys Arg Tyr Glu Ser Asn Pro Val Lys His Tyr
                1205                1210                1215

Thr Asp Met Gly Phe Thr His Asn Lys Ala Leu Glu Ala Asn Tyr Asp
            1220                1225                1230

Ala Val Lys Ala Phe Ile Asn Ala Phe Lys Lys Glu His His Gly Val
        1235                1240                1245

Asn Leu Thr Thr Arg Thr Val Leu Glu Ser Gln Gly Ser Ala Glu Leu
    1250                1255                1260

Ala Lys Lys Leu Lys Asn Thr Leu Leu Ser Leu Asp Ser Gly Glu Ser
1265                1270                1275                1280

Met Ser Phe Ser Arg Ser Tyr Gly Gly Gly Val Ser Thr Val Phe Val
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Pro Thr Leu Ser Lys Lys Val Pro Val Pro Val Ile Pro Gly Ala Gly
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Ile Thr Leu Asp Arg Ala Tyr Asn Leu Ser Phe Ser Arg Thr Ser Gly
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Gly Leu Asn Val Ser Phe Gly Arg Asp Gly Gly Val Ser Gly Asn Ile
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Met Val Ala Thr Gly His Asp Val Met Pro Tyr Met Thr Gly Lys Lys
1345 1350 1355 1360

Thr Ser Ala Gly Asn Ala Ser Asp Trp Leu Ser Ala Lys His Lys Ile
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Ser Pro Asp Leu Arg Ile Gly Ala Ala Val Ser Gly Thr Leu Gln Gly
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Thr Leu Gln Asn Ser Leu Lys Phe Lys Leu Thr Glu Asp Glu Leu Pro
1395 1400 1405

Gly Phe Ile His Gly Leu Thr His Gly Thr Leu Thr Pro Ala Glu Leu
1410 1415 1420

Leu Gln Lys Gly Ile Glu His Gln Met Lys Gln Gly Ser Lys Leu Thr
1425 1430 1435 1440

Phe Ser Val Asp Thr Ser Ala Asn Leu Asp Leu Arg Ala Gly Ile Asn
1445 1450 1455

Leu Asn Glu Asp Gly Ser Lys Pro Asn Gly Val Thr Ala Arg Val Ser
1460 1465 1470

Ala Gly Leu Ser Ala Ser Ala Asn Leu Ala Ala Gly Ser Arg Glu Arg
1475 1480 1485

Ser Thr Thr Ser Gly Gln Phe Gly Ser Thr Thr Ser Ala Ser Asn Asn
1490 1495 1500

Arg Pro Thr Phe Leu Asn Gly Val Gly Ala Gly Ala Asn Leu Thr Ala
1505 1510 1515 1520

Ala Leu Gly Val Ala His Ser Ser Thr His Glu Gly Lys Pro Val Gly
1525 1530 1535

Ile Phe Pro Ala Phe Thr Ser Thr Asn Val Ser Ala Ala Leu Ala Leu

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          1540                1545                1550

Asp Asn Arg Thr Ser Gln Ser Ile Ser Leu Glu Leu Lys Arg Ala Glu
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Pro Val Thr Ser Asn Asp Ile Ser Glu Leu Thr Ser Thr Leu Gly Lys
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His Phe Lys Asp Ser Ala Thr Thr Lys Met Leu Ala Ala Leu Lys Glu
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Leu Asp Asp Ala Lys Pro Ala Glu Gln Leu His Ile Leu Gln Gln His
               1605                1610                1615

Phe Ser Ala Lys Asp Val Val Gly Asp Glu Arg Tyr Glu Ala Val Arg
          1620                1625                1630

Asn Leu Lys Lys Leu Val Ile Arg Gln Gln Ala Ala Asp Ser His Ser
       1635                1640                1645

Met Glu Leu Gly Ser Ala Ser His Ser Thr Thr Tyr Asn Asn Leu Ser
   1650                1655                1660

Arg Ile Asn Asn Asp Gly Ile Val Glu Leu Leu His Lys His Phe Asp
1665                1670                1675                1680

Ala Ala Leu Pro Ala Ser Ser Ala Lys Arg Leu Gly Glu Met Met Asn
               1685                1690                1695

Asn Asp Pro Ala Leu Lys Asp Ile Ile Lys Gln Leu Gln Ser Thr Pro
          1700                1705                1710

Phe Ser Ser Ala Ser Val Ser Met Glu Leu Lys Asp Gly Leu Arg Glu
       1715                1720                1725

Gln Thr Glu Lys Ala Ile Leu Asp Gly Lys Val Gly Arg Glu Glu Val
   1730                1735                1740

Gly Val Leu Phe Gln Asp Arg Asn Asn Leu Arg Val Lys Ser Val Ser
1745                1750                1755                1760

Val Ser Gln Ser Val Ser Lys Ser Glu Gly Phe Asn Thr Pro Ala Leu
               1765                1770                1775

Leu Leu Gly Thr Ser Asn Ser Ala Ala Met Ser Met Glu Arg Asn Ile
          1780                1785                1790

Gly Thr Ile Asn Phe Lys Tyr Gly Gln Asp Gln Asn Thr Pro Arg Arg
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       1795                1800                1805

Phe Thr Leu Glu Gly Gly Ile Ala Gln Ala Asn Pro Gln Val Ala Ser
   1810                1815                1820

Ala Leu Thr Asp Leu Lys Lys Glu Gly Leu Glu Met Lys Ser
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<211> 420
<212> DNA
<213> Erwinia amylovora

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gctgacccac aaacttcaat aaccctgtat tcgatgctat tacagctgaa ttttgaaatg 240
gcggccatgc gcggctgttg gctggcgctg gatgaactgc acaacgtgcg tttatgtttt 300
cagcagtcgc tggagcatct ggatgaagca agttttagcg atatcgttag cggcttcatc 360
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<210> 10
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<212> PRT
<213> Erwinia amylovora

<400> 10
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Tyr Asn Glu Gln Asp Glu Glu Ala Ala Val Leu Glu Val Pro Gln His
         35                  40                  45

Ser Asp Ser Leu Leu Leu His Cys Arg Ile Ile Glu Ala Asp Pro Gln
     50                  55                  60

Thr Ser Ile Thr Leu Tyr Ser Met Leu Leu Gln Leu Asn Phe Glu Met
 65                  70                  75                  80

Ala Ala Met Arg Gly Cys Trp Leu Ala Leu Asp Glu Leu His Asn Val
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Arg Leu Cys Phe Gln Gln Ser Leu Glu His Leu Asp Glu Ala Ser Phe
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Tyr Ile Ala Gln Leu Asp Glu Ser Ser Ala Ala
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<210> 11
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<212> PRT
<213> Pseudomonas syringae

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Ser Lys Ala Leu Gln Glu Val Val Val Lys Leu Ala Glu Glu Leu Met
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Arg Asn Gly Gln Leu Asp Asp Ser Ser Pro Leu Gly Lys Leu Leu Ala
     50                  55                  60

Lys Ser Met Ala Ala Asp Gly Lys Ala Gly Gly Gly Ile Glu Asp Val
 65                  70                  75                  80

Ile Ala Ala Leu Asp Lys Leu Ile His Glu Lys Leu Gly Asp Asn Phe
                 85                  90                  95

Gly Ala Ser Ala Asp Ser Ala Ser Gly Thr Gly Gln Gln Asp Leu Met
            100                 105                 110

Thr Gln Val Leu Asn Gly Leu Ala Lys Ser Met Leu Asp Asp Leu Leu
        115                 120                 125

Thr Lys Gln Asp Gly Gly Thr Ser Phe Ser Glu Asp Asp Met Pro Met
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Leu Asn Lys Ile Ala Gln Phe Met Asp Asp Asn Pro Ala Gln Phe Pro
145                 150                 155                 160

Lys Pro Asp Ser Gly Ser Trp Val Asn Glu Leu Lys Glu Asp Asn Phe
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Leu Asp Gly Asp Glu Thr Ala Ala Phe Arg Ser Ala Leu Asp Ile Ile
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Gly Gln Gln Leu Gly Asn Gln Gln Ser Asp Ala Gly Ser Leu Ala Gly
        195                 200                 205

Thr Gly Gly Gly Leu Gly Thr Pro Ser Ser Phe Ser Asn Asn Ser Ser
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Val Met Gly Asp Pro Leu Ile Asp Ala Asn Thr Gly Pro Gly Asp Ser
225                 230                 235                 240

Gly Asn Thr Arg Gly Glu Ala Gly Gln Leu Ile Gly Glu Leu Ile Asp
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Arg Gly Leu Gln Ser Val Leu Ala Gly Gly Gly Leu Gly Thr Pro Val
            260                 265                 270

Asn Thr Pro Gln Thr Gly Thr Ser Ala Asn Gly Gly Gln Ser Ala Gln
        275                 280                 285

Asp Leu Asp Gln Leu Leu Gly Gly Leu Leu Leu Lys Gly Leu Glu Ala
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Thr Leu Lys Asp Ala Gly Gln Thr Gly Thr Asp Val Gln Ser Ser Ala
305                 310                 315                 320

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<210> 12
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<213> Pseudomonas syringae

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<210> 13
<211> 1729
<212> DNA
<213> Pseudomonas syringae

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<213> Pseudomonas syringae

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             20                  25                  30

Glu Gln Asn Thr Gln Gln Ala Ile Asp Pro Ser Ala Leu Leu Phe Gly
         35                  40                  45

Ser Asp Thr Gln Lys Asp Val Asn Phe Gly Thr Pro Asp Ser Thr Val
     50                  55                  60

Gln Asn Pro Gln Asp Ala Ser Lys Pro Asn Asp Ser Gln Ser Asn Ile
 65                  70                  75                  80

Ala Lys Leu Ile Ser Ala Leu Ile Met Ser Leu Leu Gln Met Leu Thr
                 85                  90                  95

Asn Ser Asn Lys Lys Gln Asp Thr Asn Gln Glu Gln Pro Asp Ser Gln
            100                 105                 110

Ala Pro Phe Gln Asn Asn Gly Gly Leu Gly Thr Pro Ser Ala Asp Ser
        115                 120                 125

Gly Gly Gly Gly Thr Pro Asp Ala Thr Gly Gly Gly Gly Gly Asp Thr
    130                 135                 140

Pro Ser Ala Thr Gly Gly Gly Gly Gly Asp Thr Pro Thr Ala Thr Gly
145                 150                 155                 160

Gly Gly Gly Ser Gly Gly Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly
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Ser Gly Gly Thr Pro Thr Ala Thr Gly Gly Gly Glu Gly Gly Val Thr
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Pro Gln Ile Thr Pro Gln Leu Ala Asn Pro Asn Arg Thr Ser Gly Thr
        195                 200                 205

Gly Ser Val Ser Asp Thr Ala Gly Ser Thr Glu Gln Ala Gly Lys Ile
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Asn Val Val Lys Asp Thr Ile Lys Val Gly Ala Gly Glu Val Phe Asp
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Gln Gly Glu Asn Gln Lys Pro Met Phe Glu Leu Ala Glu Gly Ala Thr
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Leu Lys Asn Val Asn Leu Gly Glu Asn Glu Val Asp Gly Ile His Val
        275                 280                 285

Lys Ala Lys Asn Ala Gln Glu Val Thr Ile Asp Asn Val His Ala Gln
    290                 295                 300

Asn Val Gly Glu Asp Leu Ile Thr Val Lys Gly Glu Gly Gly Ala Ala
305                 310                 315                 320

Val Thr Asn Leu Asn Ile Lys Asn Ser Ser Ala Lys Gly Ala Asp Asp
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Lys Val Val Gln Leu Asn Ala Asn Thr His Leu Lys Ile Asp Asn Phe
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Lys Ala Asp Asp Phe Gly Thr Met Val Arg Thr Asn Gly Gly Lys Gln
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Phe Asp Asp Met Ser Ile Glu Leu Asn Gly Ile Glu Ala Asn His Gly
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Lys Phe Ala Leu Val Lys Ser Asp Ser Asp Asp Leu Lys Leu Ala Thr
385                 390                 395                 400

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<210> 15
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<213> Pseudomonas solanacearum

<400> 15

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            20                  25                  30

Val Gln Asp Leu Ile Lys Gln Val Glu Lys Asp Ile Leu Asn Ile Ile
        35                  40                  45

Ala Ala Leu Val Gln Lys Ala Ala Gln Ser Ala Gly Gly Asn Thr Gly
    50                  55                  60

Asn Thr Gly Asn Ala Pro Ala Lys Asp Gly Asn Ala Asn Ala Gly Ala
65                  70                  75                  80

Asn Asp Pro Ser Lys Asn Asp Pro Ser Lys Ser Gln Ala Pro Gln Ser
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Ala Asn Lys Thr Gly Asn Val Asp Asp Ala Asn Asn Gln Asp Pro Met
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Gln Ala Leu Met Gln Leu Leu Glu Asp Leu Val Lys Leu Leu Lys Ala
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Ala Leu His Met Gln Gln Pro Gly Gly Asn Asp Lys Gly Asn Gly Val
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Gly Gly Ala Asn Gly Ala Lys Gly Ala Gly Gly Gln Gly Gly Leu Ala
145                 150                 155                 160

Glu Ala Leu Gln Glu Ile Glu Gln Ile Leu Ala Gln Leu Gly Gly Gly
                165                 170                 175

Gly Ala Gly Ala Gly Gly Ala Gly Gly Gly Val Gly Gly Ala Gly Gly
            180                 185                 190

Ala Asp Gly Gly Ser Gly Ala Gly Gly Ala Gly Gly Ala Asn Gly Ala
        195                 200                 205

Asp Gly Gly Asn Gly Val Asn Gly Asn Gln Ala Asn Gly Pro Gln Asn
    210                 215                 220

Ala Gly Asp Val Asn Gly Ala Asn Gly Ala Asp Asp Gly Ser Glu Asp
225                 230                 235                 240

Gln Gly Gly Leu Thr Gly Val Leu Gln Lys Leu Met Lys Ile Leu Asn
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        275                 280                 285

Ala Asn Pro Gly Ala Asn Gln Pro Gly Ser Ala Asp Asp Gln Ser Ser
    290                 295                 300

Gly Gln Asn Asn Leu Gln Ser Gln Ile Met Asp Val Val Lys Glu Val
305                 310                 315                 320

Val Gln Ile Leu Gln Gln Met Leu Ala Ala Gln Asn Gly Gly Ser Gln
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<400> 16

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<210> 17
<211> 10
<212> PRT

<213> Xanthomonas campestris

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<210> 18
<211> 20
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<213> Xanthomonas campestris pv. pelargonii

<400> 18
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1 5 10 15

Leu Leu Ala Met
20

HARPIN *FROM ERWINIA AMYLOVORA* INDUCES PLANT RESISTANCE

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Plants have evolved a complex array of biochemical pathways that enable them to recognize and respond to signals from the environment. A common form of plant resistance is the restriction of pathogen proliferation to a small zone surrounding the site of infection. Typically this restriction is accompanied by localized necrosis. In addition to local defense response, plants also respond to infection by activating defenses in uninfected parts of the plant, which result in resistance of the plant to secondary infection (Dean and Kuc, 1985). Collectively, this phenomenon of induced resistance is called systemic acquired resistance (SAR). SAR reduces the severity of disease caused by all classes of pathogens and it can persist for several weeks or longer. SAR can be induced by abiotic agents, such as salicylic acid as well as biotic agents, such as virulent and avirulent pathogens (Dean and Kuc, 1985; Malamy *et al.*, 1990). Salicylic acid is believed to play a signal function in the induction of SAR since endogenous levels of salicylic acid increase after "immunization" with an incompatible pathogen. However at present, little is known about the signal transduction pathways activated during responses of a plant to attack by a pathogen, although this knowledge is central to understanding disease susceptibility and resistance.

Erwinia amylovora is an often devastating plant pathogenic bacterium that causes the fire blight disease of pear, apple and many other rosaceous plants. In non-host plants, *E. amylovora* elicits the hypersensitive response (HR), which is characterized by a rapid, localized death of tissues infiltrated with high concentrations of bacterial cells ($>10^7$ cfu/ml) (Klement, 1982). *hrp* genes are essential for *E. amylovora* to cause disease in host plants and to elicit the HR in non-host plants (Beer *et al.*, 1991). Harpin is a heat-stable, glycine-rich, secreted protein with molecular mass of 37 kD. It is encoded by *hrpN* of *E. amylovora* (Wei *et al.*, 1992). When infiltrated into intercellular spaces, harpin elicits the HR in many plants including tobacco, pepper, sunflower, tomato cabbage, arabidopsis, cucumber, geranium, watermelon and lettuce.

The HR is believed to be associated with plant defense against pathogens. Hence, we reasoned that harpin-induced HR may induce plant resistance. We tested harpin-induced resistance in more than seven different plants against eight diseases caused by fungi, bacteria and viruses. All tested plants showed some resistance. Here we report evidence of harpin-induced resistance to three diseases, southern bacterial wilt of tomato, tobacco mosaic virus and Gliocladium leaf spot of cucumber.

Harpin-induced resistance in tomato against southern bacterial wilt caused by *Pseudomonas solanacearum*.

100 μl of a cell suspension of ca. 10^8 cfu/ml of *Escherichia coli* DH5α(pCPP430) or 100 µl of a 200 µg/ml crude harpin preparations were infiltrated into portions of the two lower true leaves of two-week-old tomato seedlings grown in 8 x 15 cm flats in the greenhouse. Twenty plants were used for each treatment. Necrosis was evident 24 hours after infiltration of harpin or *E. coli* DH5α(pCPP430), which produces and secretes

harpin. Four days after the tomato seedlings had been treated with harpin or bacteria, they were inoculated with *P. solanacearum* K60 (10^7 cfu/ml) by root dipping for three minutes. The inoculated plants were replanted into the same flats and left in a greenhouse. None of the 20 harpin-infiltrated plants showed any symptoms one week after inoculation with *P. solanacearum* K60. However, seven of the 20 buffer-infiltrated plants were stunted. After two weeks, 11 buffer-infiltrated plants showed severe wilting and five were stunted, characteristics of the southern bacterial wilt disease. In comparison, only two harpin-treated plants appeared wilted and three plants were stunted. Similar induced resistance was observed following infiltration of living bacteria *E. coli*/DH5α(pCPP430), but not by *E. coli* DH5α(pCPP430$^-$), which is a harpin-deficient mutant created by transposon Tn*5tac* insertion into the *hrpN* gene. These results indicate that harpin, which is produced and secreted by *hrp* gene cluster of *E. amylovora*, is responsible for the induced-resistance realized.

Harpin-induced resistance in tobacco to tobacco mosaic virus (TMV)

One panel of a lower leaf of four-week-old tobacco seedlings (*Nicotiana tabacum* L. "Xanthi" with *N* gene) was infiltrated with 100 µl of a 200 µg/ml crude harpin preparation in 5 mM phosphate buffer. Three days later, the plants were challenged with TMV. Fifty µl of a suspension of TMV (5 µg/ml) was rubbed on one upper leaf with 400-mesh carborundum. Six plants were used for each treatment. Necrotic lesions appeared on inoculated leaves of both harpin- and buffer-treated plants 4 days after inoculation. The average number of necrotic lesions from the six harpin-treated plants was 21, which was significantly less than the 67 lesion average that developed on six buffer-treated plants. More importantly, the size of the lesions on buffer-treated plants was larger than those on the harpin-treated plants. Actually, it was difficult to distinguish individual lesions on the buffer-treated plants by day 10, because several necrotic lesions had merged.

Harpin-induced resistance against Gliocladium leaf spot of cucumber

Harpin or a cell suspension of *E. coli* DH5α(pCPP430) was infiltrated into first two true leaves of two-week-old cucumber seedlings. Six plants were infiltrated for each treatment. Four days after infiltration of harpin, a *Gliocladium cucurbitae* spore suspension (10^6 spores/ml) was sprayed onto the whole plants. The inoculated plants were incubated in a moisture chamber. Ten days after the inoculation, typical leaf spots appeared. A mean of six lesions was present on the lowest leaves of six harpin-treated plants, but 32 lesions formed on the same leaves of the six buffer-treated plants. On the third lowest leaves, the difference in disease severity was even greater; there were virtually no lesions on harpin-treated plants, however, more than 30 lesions were found on the buffer-treated plants. Later, most of the diseased leaves on buffer-treated plants wilted and died.

The examples outlined above show that harpin is able to induce resistance in different plants against bacterial, viral and fungal pathogens. Although mechanisms of harpin-induced resistance are unknown, some of our preliminary experiments have shown that harpin may act as an elicitor of salicylic acid induction, which is believed to be involved in SAR (Malamy *et al.*, 1990). Unlike some host-specific elicitors (Keen *et al.*, 1990), harpin is able to elicit the HR on a broad range of plants. Thus, we expect that harpin-induced resistance can be achieved in many plants either by manipulation of harpin exogenously or by harpin-mediated transgenic plants.

Our studies of harpin-induced resistance are just beginning and we need to learn more to understand the exciting features of this phenomenom. For example, what is the minimal amount of harpin needed to induce plant resistance and how long does the resistance persist, and what mechanisms are involved in harpin-induced resistance? We expect that harpin as a novel molecule will play an important role in dissecting the signal transduction pathways of induced-resistance in plants, and perhaps also in practical disease control.

ACKNOWLEDGEMENT

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Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 $HrpZ_{Pss}$ protein

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Summary

Systemic acquired resistance (SAR) is an inducible plant defense response and is effective against a broad spectrum of pathogens. Biological induction of SAR usually follows plant cell death resulting from the plant hypersensitive response (HR) elicited by an avirulent pathogen or from disease necrosis caused by a virulent pathogen. The elicitation of the HR and disease necroses by pathogenic bacteria is controlled by *hrp* genes. Previously, it was shown that the *Pseudomonas syringae* 61 (Pss61) $HrpZ_{Pss}$ protein (formally $harpin_{Pss}$) elicited the HR in plants. In this study, it is shown that $HrpZ_{Pss}$ induced SAR in cucumber to diverse pathogens, including the anthracnose fungus (*Colletotrichum lagenarium*), tobacco necrosis virus and the bacterial angular leaf spot bacterium (*P. s.* pv. *lachrymans*). A *hrpH* mutant of Pss61, which is defective in the secretion of $HrpZ_{Pss}$ and, possibly, other protein elicitors, failed to elicit SAR. Pathogenesis-related (PR) proteins, including peroxidase, β-glucanase and chitinases, were induced in cucumber plants inoculated with Pss61, *C. lagenarium* or $HrpZ_{Pss}$. The induction patterns of PR proteins by $HrpZ_{Pss}$ and Pss61 were the same, but were different from that induced by *C. lagenarium*. Interestingly, the *hrpH* mutant induced two of the three identified PR proteins, despite its failure to induce SAR. These results suggest that proteinaceous elicitors, such as $HrpZ_{Pss}$, that traverse the bacterial Hrp secretion pathway are involved in the biological induction of SAR and that at least some PR proteins can be induced by bacterial factors that are not controlled by *hrp* genes.

Introduction

Localized infection of plants by necrotizing pathogens can result in systemic acquired resistance (SAR) to disease, which persists for weeks to months and is effective against diverse pathogens including fungi, bacteria, and necrotizing viruses (Kuc, 1982; Ross, 1961). Biological induction of SAR is usually associated with prior plant cell death during the hypersensitive response (HR) or disease necrosis triggered by avirulent or virulent pathogens, respectively (Cameron *et al.*, 1994; Kuc, 1982; Ross, 1961; Uknes *et al.*, 1993). Certain synthetic chemicals, such as salicylic acid (SA) and 2,6-dichloroisonicotinic acid (INA), also can be very effective in the induction of SAR when applied to plants (Metraux *et al.*, 1991; White, 1979). The induction of SAR in cucumber plants by an avirulent bacterial pathogen, *Pseudomonas syringae* pv. *syringae*, appears to be dependent on bacterial *hrp* genes that are required for many plant pathogenic bacteria to elicit the HR in non-host plants or to cause disease in host plants (Smith *et al.*, 1991). The HR is a complex plant resistance reaction which involves local plant cell death and restriction of pathogens to the site of their introduction (Klement, 1982).

Recent studies have shown that most Hrp proteins are involved in the assembly of a type III protein secretion pathway (the Hrp pathway) through which bacterial pathogenesis-related proteins traverse to the extracellular milieu to initiate various plant–bacterial interactions (Fenselau, 1992; Huang *et al.*, 1992, 1995; Van Gijsegem *et al.*, 1995). One family of such proteins that have been identified are heat-stable, glycine-rich proteins: harpin of *Erwinia amylovora* (Wei *et al.*, 1992), $HrpZ_{Pss}$ (formally $harpin_{Pss}$) of *P. s.* pv. *syringae* 61 (Pss61) (He *et al.*, 1993) and PopA of *P. solanacearum* (Arlat *et al.*, 1994). Harpins and PopA were shown to elicit the HR when infiltrated into the leaf laminae of appropriate plants (Arlat *et al.*, 1994; He *et al.*, 1993; Wei *et al.*, 1992), to induce exchange of H^+ and K^+ (the 'XR') across the plasmalemma (Wei *et al.*, 1992), and to generate active oxygen species (Baker *et al.*, 1993) when added to plant cell cultures, which are all properties of the HR elicited by live bacteria.

As part of our investigation into plant responses to *P. syringae* extracellular proteins under the control of the Hrp regulatory/secretion system, we studied the involvement of $HrpZ_{Pss}$ in the biological induction of SAR by *P. s.* pv. *syringae* 61. In this paper we describe the experimental results showing that $HrpZ_{Pss}$, as well as the bacterium (Pss61) that produces it, efficiently induced SAR in cucumber to diverse pathogens, including a fungus (*Colletotrichum lagenarium*), a bacterium (*P. s.* pv. *lachrymans*) and a local lesion-forming virus (tobacco necrosis virus). The *hrpH* mutant, which is defective in the secretion of $HrpZ_{Pss}$, failed to induce SAR. Multiple pathogenesis-related (PR) proteins were detected in cucumber plants treated with $HrpZ_{Pss}$, Pss61 and *C. lagenarium*. The efficacy

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of SAR induction, resistance spectrum and patterns of PR protein induction were very similar in plants treated with $HrpZ_{Pss}$ and Pss61. Interestingly, the PR protein patterns induced by $HrpZ_{Pss}$ and Pss61 were somewhat different from that induced by *C. lagenarium*. The *hrpH* mutant, though unable to induce SAR, efficiently induced some of the well-characterized PR proteins. These results suggest that the biological induction of SAR by *P. syringae* is dependent on the bacterial proteins (such as $HrpZ_{Pss}$) which traverse the Hrp secretion pathway and that at least some PR-proteins can be induced by bacterial factors other than Hrp-controlled extracellular proteins.

Results

Symptoms on cucumber leaves treated with SAR inducers

Treatment of leaves with spores of *C. lagenarium* (a virulent, necrogenic pathogen of cucumber) resulted in the development of symptoms typically obtained with the fungus in cucumber: infiltrated areas were asymptomatic for 3–4 days, after which time tissues began to collapse and become necrotic. Lesions continued to expand for several days and developed a tan to brown pigmentation. Symptoms induced by treatments with Pss61 (an avirulent, HR necrosis-inducing pathogen) and $HrpZ_{Pss}$ varied with environmental conditions in the greenhouse. Under high levels of natural light, Pss61 and $HrpZ_{Pss}$ triggered the HR within 24 and 48 h, respectively, after infiltration. The HR was restricted to infiltrated areas and did not expand as did the necroses caused by *C. lagenarium*. Under lower natural light levels (cloudy days), tissues infiltrated with Pss61 or $HrpZ_{Pss}$ developed a weaker HR characterized by increasing chlorosis over a 3–5 day period, then necroses developed gradually and irregularly, despite supplemental illumination with sodium lamps. Infiltration with *hrpH* (which is defective in the secretion of $HrpZ_{Pss}$, He *et al.*, 1993) caused either no symptoms or a very mild chlorosis under all conditions tested. Infiltration with buffer alone caused only a small ring of white necrosis resulting from mechanical damage caused by pressure of the pipette mouth against the leaf. Interestingly, infiltration with *E. amylovora* harpin protein, which was prepared from DH5α(pCPP50) (He *et al.*, 1994) and which induced a strong HR in tobacco leaves, did not induce HR necrosis in cucumber leaves (data not shown).

SAR to C. lagenarium

We first tested to see whether $HrpZ_{Pss}$ alone could induce SAR to a well-studied fungal pathogen of cucumber, *C. lagenarium*. As shown in Table 1, $HrpZ_{Pss}$ treatment induced SAR comparable to that induced by *C. lagenarium* (approximately 90% reduction in total necrotic area relative to buffer-treated controls) in two upper leaves which expanded subsequent to induction treatment. The degrees of SAR induced by $HrpZ_{Pss}$, Pss61, Pss61-*hrpH* and *C. lagenarium* in cucumber were subsequently compared. Under conditions conducive to HR development in the greenhouse (high levels of natural light due to sunny weather) both $HrpZ_{Pss}$ and Pss61 efficiently induced SAR in Leaf 2 and Leaf 3 (Table 2 and Figure 1a and b). SAR was expressed as a reduction in both the number and diameter of necrotic lesions resulting from challenge with *C. lagenarium*. Protection of Leaf 2 was comparable to that induced by *C. lagenarium*, whereas protection in Leaf 3 was weaker than that induced by the fungus. Under the conditions of this experiment, expansion of Leaf 2 and Leaf 3 occurred after the onset of the HR and necrosis incited by *C. lagenarium* infiltration. Leaf 2 was fully expanded prior to challenge-inoculation, whereas Leaf 3 was not. The *hrpH* mutant did not induce SAR (Table 2). The quality and/or quantity of light profoundly influenced the induction of both the HR and SAR in cucumber by Pss61 and $HrpZ_{Pss}$ in the greenhouse. When a similar experiment was conducted under conditions non-conducive to HR development (low levels of natural light on cloudy days), neither Pss61 nor $HrpZ_{Pss}$ induced the HR or SAR, although *C. lagenarium* incited necrotic lesions on Leaf 1 and induced SAR under these conditions (data not shown).

SAR to TNV

We next examined whether $HrpZ_{Pss}$-induced SAR would be effective against a viral pathogen. In two initial experiments, the abilities of $HrpZ_{Pss}$ and *C. lagenarium* to induce SAR to TNV were compared. $HrpZ_{Pss}$ elicited a normal HR in these experiments and induced SAR to TNV local lesion formation comparable to that induced by *C. lagenarium* (Table 3 and Figure 1c and d). We then compared the abilities of $HrpZ_{Pss}$, Pss61, *hrpH*, and *C. lagenarium* to induce SAR to TNV. Under high light conditions, $HrpZ_{Pss}$ and Pss61 elicited a normal HR and induced SAR which restricted local lesion formation by TNV to an extent similar to that of SAR induced by *C. lagenarium*. The percentage of lesion number reduction was 68% for Pss61, 67.1% for $HrpZ_{Pss}$, and 75.5% for *C. lagenarium* (Table 3). Under low natural light conditions unfavorable for HR development (see Experimental procedures), $HrpZ_{Pss}$ and Pss61 elicited a weaker degree of SAR relative to that induced by *C. lagenarium*. The percentage of lesion number reduction was 44.9% for Pss61, 46.7% for $HrpZ_{Pss}$, and 89.6% for *C. lagenarium* (Table 3). The lesion numbers observed in these independent experiments varied greatly, mainly due to the use of different TNV inoculum preparations. TNV inoculum was prepared freshly each time from cucumber

Table 1. Induction by HrpZ$_{Pss}$ and the fungal pathogen, *C. lagenarium*, of systemic acquired resistance to *C. lagenarium* in cucumber

Treatment	Leaf 2			Leaf 3		
	Lesion number	Lesion diameter (mm)	Total necrotic area (mm^2)	Lesion number	Lesion diameter (mm)	Total necrotic area (mm^2)
Buffer	18.8 ± 0.6[a]	2.0 ± 0.1	60.9 ± 7.4	18.5 ± 0.6	2.5 ± 0.3	110.2 ± 29.0
HrpZ$_{Pss}$	6.5 ± 0.9	1.1 ± 0.0	6.9 ± 1.3	9.5 ± 1.7	1.3 ± 0.1	13.4 ± 3.7
C. lagenarium	3.3 ± 0.8	1.0 ± 0.0	2.6 ± 0.6	6.5 ± 1.3	1.2 ± 0.1	7.5 ± 1.3

[a]Mean + SE of four replicate plants per treatment.
Leaf 1 of young plants was infiltrated with buffer (5 mM $MgSO_4$), or HrpZ$_{Pss}$ (80 μg ml^{-1}) in buffer, or spores of *C. lagenarium* (5×10^4 spores ml^{-1}). After 7 days, Leaf 2 and Leaf 3 were challenged with 20 droplets per leaf containing spores of *C. lagenarium*. Disease was allowed to develop for 8 days.

Table 2. Induction of systemic acquired resistance to *C. lagenarium* in cucumber by *P. s.* pv. *syringae* 61 (Pss61), HrpZ$_{Pss}$, the *hrpH* mutant of Pss61 and *C. lagenarium*

Treatment	Leaf 2			Leaf 3		
	Lesion number	Lesion diameter (mm)	Total necrotic area (mm^2)	Lesion number	Lesion diameter (mm)	Total necrotic area (mm^2)
Buffer	15.4±1.2[a]	1.6±0.2	38.9±8.3	16.2±1.0	1.8±0.1	52.0±8.1
hrpH	13.2±1.1	1.7±0.1	32.1±2.2	15.4±1.6	1.8±0.1	50.0±11.9
Pss61	5.4±0.4	1.2±0.1	7.0±1.9	9.4±1.1	1.5±0.1	21.2±6.2
HrpZ$_{Pss}$	5.0±0.5	1.2±0.1	5.9±1.4	8.6±2.5	1.6±0.2	24.4±9.1
C. lagenarium	4.0±1.2	1.3±0.3	8.4±5.3	6.4±1.4	1.4±0.2	13.2±5.0

[a]Mean ± SE of five replicate plants per treatment.
Leaf 1 of young plants was infiltrated with buffer (5 mM $MgSO_4$), bacteria (OD_{600}=0.2), HrpZ$_{Pss}$ (160 μg ml^{-1}), or spores of *C. lagenarium* (5×10^4 ml^{-1}). After 8 days, Leaf 2 and Leaf 3 were challenged with 20 droplets per leaf containing spores of *C. lagenarium*. Disease was allowed to develop for 8 days.

leaves bearing TNV lesions. In experiment 3, the *hrpH* mutant induced a low level of SAR to TNV (Table 3).

SAR to P. syringae *pv.* lacrymans

HrpZ$_{Pss}$ and *C. lagenarium* also induced SAR to the angular leaf spot bacterium, *P. s.* pv. *lacrymans*. For these experiments, cucumber plants were challenge-inoculated at 11 days (by spraying) or 17 days (by rubbing) after treatment of Leaf 1 (Table 4). Although *C. lagenarium* was a more effective treatment, HrpZ$_{Pss}$ also induced significant levels of SAR to the bacterium, reducing necrotic lesion numbers by 32 and 75%, compared with 50 and 86% for *C. lagenarium*, in the two experiments, respectively.

Induction of PR proteins

PR proteins that accumulated in treated cucumber plants were first analyzed using native polyacrylamide gel electrophoresis (PAGE). All treatments (*C. lagenarium*, Pss61 and HrpZ$_{Pss}$) that induced SAR also induced the accumulation of three PR protein bands (tentatively named PR-A, PR-B and PR-C) (Figure 2a). *C. lagenarium* induced PR-C, but not PR-A and PR-B, in systemic leaves, while Pss61 and HrpZ$_{Pss}$ induced PR-B, but not PR-A and PR-C, in systemic leaves. Treatment with buffer or *hrpH* mutant did not induce these particular PR protein bands to levels that would allow visual identification. To see whether any PR proteins with known functions were induced in these plants, protein extracts were analyzed using native PAGE coupled with enzyme (chitinase, peroxidase and β-glucanase) activity staining. As shown in Figure 2(b), all three enzymes were induced in plants treated with HrpZ$_{Pss}$, Pss61 or *C. lagenarium* in both local (treated) and systemic leaves, although induction of chitinase isoforms by Pss61 and HrpZ$_{Pss}$ in systemic leaves was variable and low. The enzyme activities were substantially higher in local leaves than in systemic leaves. Surprisingly, although the *hrpH* mutant bacterium failed to induce SAR, it efficiently induced peroxidase and chitinase, especially in treated leaves (Figure 2b). Only β-glucanase was not found to be induced to high levels in the *hrpH*-treated plants (Figure 2b). It is interesting to note that PR protein levels induced by various treatments correlated well with degrees of SAR induced by the same treatments (*C. lagenarium* >HrpZ$_{Pss}$=Pss61>*hrpH*> or = buffer).

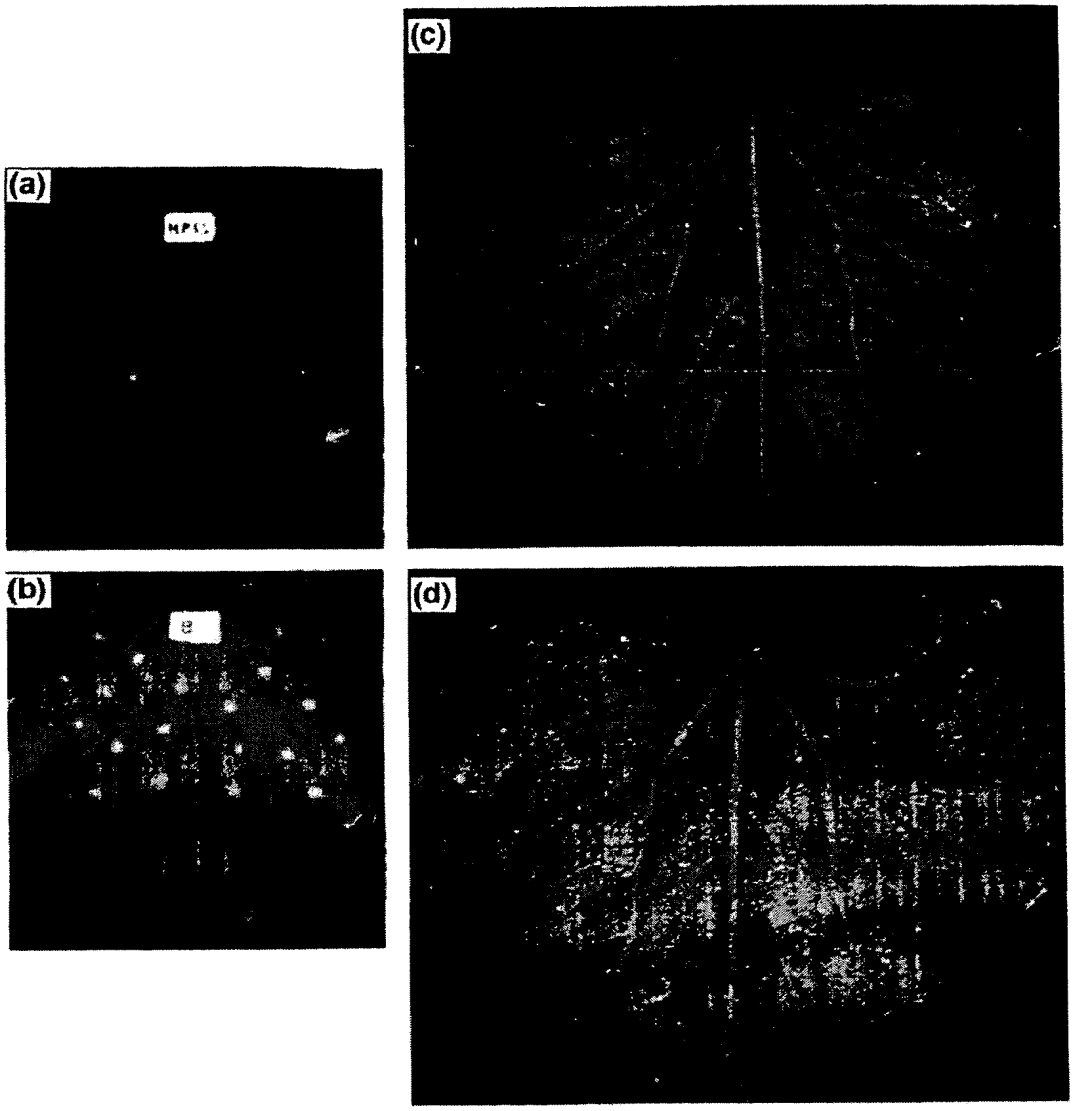

Figure 1. Disease symptoms caused by challenge-infection of *C. lagenarium* and tobacco necrosis virus on cucumber leaves with or without prior induction of SAR.
Anthracnose symptoms on Leaf 2 of cucumber plants with Leaf 1 previously treated with $HrpZ_{Pss}$ (80 μg ml^{-1}, a) or buffer (5 mM $MgSO_4$, b). Leaf 1 of young plants was infiltrated with buffer or $HrpZ_{Pss}$. After 8 days, Leaf 2 and Leaf 3 were challenged with 20 droplets per leaf containing spores of *C. lagenarium*. Disease was allowed to develop for 8 days, when the picture was taken.
TNV symptoms on Leaf 3 of cucumber plants with Leaf 1 previously treated with $HrpZ_{Pss}$ (c) or buffer (d). Leaf 1 was treated by infiltration of buffer or $HrpZ_{Pss}$ as described in footnotes to Table 1. After 7 days, Leaf 3 was challenged by mechanical inoculation with a TNV suspension prepared from infected cucumber leaves. Disease was allowed to develop for 9 days,when the picture was taken.

Induction of the pr-1 *gene and SAR in tobacco*

$HrpZ_{Pss}$ also induced SAR to tobacco mosaic virus (TMV) in tobacco (Table 5). The SAR level induced by $HrpZ_{Pss}$ was less than that induced by TMV. This was consistent with the different levels of induction of the *pr*-1 gene by $HrpZ_{Pss}$ and TMV (Figure 3). TMV-inoculated local leaves (the third and fourth true leaves) also showed more necrosis than those infiltrated with $HrpZ_{Pss}$ (data not shown), which may be partly responsible for the different levels of SAR and *pr*-1 expression in TMV- and $HrpZ_{Pss}$-induced plants.

Discussion

In this study, we show that $HrpZ_{Pss}$, a bacterial hrp gene product secreted via the Hrp pathway of *P. s.* pv. *syringae*, induced SAR in cucumber and tobacco. In cucumber, the

Table 3. Induction of systemic acquired resistance to TNV in cucumber by *hrpH* mutant, HrpZ$_{Pss}$, Pss61 and *C. lagenarium*

Treatment	Number of TNV necrotic local lesions			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Buffer	99.7±19.6[a]	47.2±0.9[b]	730.0±63.9[a]	342.8±34.3[a]
hrpH	–	–	556.0±53.4	324.3±11.2
HrpZ$_{Pss}$	28.7±3.8	7.5±1.2	240.4±27.5	182.8±18.8
Pss61	–	–	239.9±59.7	189.0±41.9
C. lagenarium	34.7±16.6	9.0±1.8	178.8±25.9	35.8±4.6

[a]Mean ± SE of three replicate plants per treatment. [b]Mean ± SE of eight replicate plants per treatment.
Leaf 1 was treated by infiltration of candidate inducers as described in the footnotes of Table 1. After 7 days, leaf 3 was challenged by mechanical inoculation with a TNV suspension prepared from infected cucumber leaves. Disease was allowed to develop for 10 or 9 days in experiments 1 and 2, respectively.
Experiments 1, 2 and 3 were performed under high levels of natural light during induction periods.
Experiment 4 was performed on cloudy days.

Table 4. Induction of systemic acquired resistance to *P. syringae* pv. *lacrymans* by HrpZ$_{Pss}$ and *C. lagenarium*

Treatment	Number of necrotic lesions[a]	
	Inoculated by rubbing	Inoculated by spraying
Buffer	244.8±34.2	56.6±5.9
HrpZ$_{Pss}$	168.5±24.5	13.8±1.7
C. lagenarium	122.8±9.8	8.3±2.1

[a]Mean ± SE of five replicate plants per treatment.
Leaf 1 of young plants was infiltrated with treatments as described in the footnotes of Table 1. Leaf 5 was challenged by rubbing, or by spraying the abaxial leaf surface with a suspension of bacterial cells (OD_{600}=0.2, 17 days after induction; or OD_{600}=0.1, 11 days after induction, respectively). Disease was allowed to develop for 7 or 13 days in rub-inoculated or spray-inoculated plants, respectively.

efficacy against fungal, viral and bacterial pathogens and persistence (for at least 17 days in the bacterial challenge experiments) of HrpZ$_{Pss}$-induced SAR is comparable to that induced by the bacterium (Pss61) that produces HrpZ$_{Pss}$. The degree of SAR induced in cucumber by HrpZ$_{Pss}$ was also comparable to that induced by a well-studied biological inducer of SAR, *C. lagenarium* (Kuc and Richmond, 1977). The *hrpH* mutant of *P. s.* pv. *syringae*, which is defective in the secretion of HrpZ$_{Pss}$ and other proteinaceous pathogenicity factors (He *et al.*, 1993; Huang *et al.*, 1992; Yuan *et al.*, in preparation), failed to induce SAR in cucumber. The induced PR protein patterns were the same in cucumber plants treated with Pss61 and HrpZ$_{Pss}$, but were different from that in *C. lagenarium*-treated plants. Moreover, the *hrpH* mutant, although unable to induce SAR, efficiently induced at least two well-characterized PR proteins, chitinase and peroxidase (Figure 2b). These results suggest that the biological induction of SAR and PR proteins by *P. s.* pv. *syringae* 61 in the non-host plant, cucumber, is dependent on the production and secretion of proteinaceous elicitors of the HR, such as HrpZ$_{Pss}$, but that at least some PR proteins can be induced by bacterial molecules independent of *hrp* gene functions.

The efficacy of both HrpZ$_{Pss}$ and Pss61 as inducers of SAR in cucumber appeared to be contingent upon their ability to elicit a normal HR, as low levels of natural light during the induction period, which interfered with HR development, resulted in reduced SAR to TNV and no SAR to *C. lagenarium* (Table 3; Strobel and He, unpublished work). The negative effect of low light likely resulted from an effect on HR development rather than upon the plant's capacity to express SAR because *C. lagenarium* formed necrotic lesions typical of this compatible pathogen on Leaf 1 (the inducer leaf) and triggered SAR under these same conditions. The profound effect of light on the development of the HR has been observed previously (Sequeira, 1979), although the underlying mechanism remains to be determined. The dependence of the induction of SAR on the HR is further suggested by our observations that the *hrpH* mutant of Pss61, which produces but does not secrete HR elicitors (He *et al.*, 1993), did not elicit the HR or induce SAR in cucumber. Furthermore, *E. amylovora* harpin, another HR elicitor which is structurally different from HrpZ$_{Pss}$ and which elicited a strong HR in tobacco, did not induce an HR or SAR in cucumber plants (Strobel and He, unpublished observation). In conclusion, there appears to be a tight linkage between HR development and induction of SAR in plants by avirulent bacteria.

The tight linkage between the HR and SAR suggests that the signal(s) for the induction of SAR by HrpZ$_{Pss}$ and *P. s.* pv. *syringae* 61 likely comes from dying plant cells and/or cells immediately adjacent to the dying cells during the HR. What types of cell death would lead to the induction of SAR? It has been shown that the HR triggered by live bacteria (Keen *et al.*, 1981), HrpZ$_{Pss}$ (He *et al.*, 1993) or *E. amylovora* harpin (He *et al.*, 1994) involves an active cell death pathway. Does this mean that only cells undergoing active cell death give rise to signals for SAR? The answer to this is probably not simple. SAR and PR proteins can

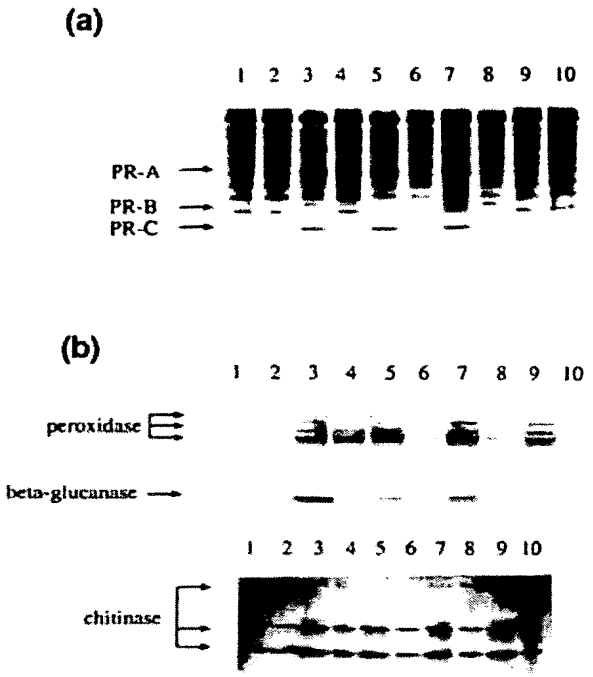

Figure 2. PR protein accumulation in cucumber plants.
PAGE (a) and PAGE coupled with activity staining (b) analyses of protein extracts from treated (lanes 1, 3, 5, 7 and 9) or systemic leaves (lanes 2, 4, 6, 8, and 10). The treatments were buffer (lanes 1 and 2), *C. lagenarium* (lanes 3 and 4), Pss61 (lanes 5 and 6), $HrpZ_{Pss}$ (lanes 7 and 8) and the *hrpH* mutant (lanes 9 and 10). PR-A, PR-B and PR-C are tentative names for the three PR proteins observed in these experiments. The identities of these PR proteins are unknown.

Table 5. Induction of systemic acquired resistance to TMV by $HrpZ_{Pss}$ and TMV

	Diameter of necrotic lesions[a]
Buffer	4.41 ± 0.05
$HrpZ_{Pss}$	3.05 ± 0.03
TMV	2.34 ± 0.03

[a]Mean ± SE of 100 lesions per treatment.
The third and fourth true leaves of 6-week-old tobacco plants were inoculated with TMV (100–150 lesions per leaf), or infiltrated with 120 μg ml^{-1} $harpin_{Pss}$ or 5 mM $MgSO_4$ at 10 sites (50 μl per site). Five days later the seventh and eighth true leaves were challenge-inoculated with TMV. The diameters of TMV lesions on the challenged leaves were recorded.

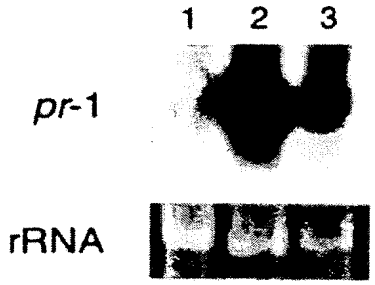

Figure 3. Induction of the *pr*-1 gene in tobacco leaves.
Total RNA was isolated from systemic leaves (the ninth true leaves) of plants treated with buffer (lane 1), TMV (lane 2), or $HrpZ_{Pss}$ (lane 3) 5 days post-induction. A PCR-amplified internal fragment of the tobacco *pr*-1 gene was labeled with [α-^{32}P]dATP and used as a probe. The largest rRNA species visualized after staining with ethidium bromide was used as a reference.

be induced not only by HR-eliciting avirulent pathogens, but also by necrosis-causing virulent pathogens. For example, *P. s.* pv. *lacrymans* and *C. lagenarium* can efficiently induce SAR and/or PR proteins in the susceptible host plant, cucumber (Kuc and Richmond, 1977; Smith *et al.*, 1991; this study). Unless cell death during the HR and some diseases shares the same biochemical processes, which is possible, the ability of both virulent and avirulent pathogens to induce SAR argues for multiple cell death pathways in the induction of SAR. On the other hand, not all types of plant cell death induce SAR. For example, cell death due to mechanical wounding or resulting from certain plant mutations does not induce SAR (Dietrich *et al.*, 1994). It would be important in the future to learn why certain cell death processes, but not others, lead to SAR. Endogenous signaling molecules, such as salicylic acid and H_2O_2, have been shown or suggested to be involved in the induction of SAR (Chen *et al.*, 1993; Gatfney *et al.*, 1993; Malamy *et al.*, 1990; Metraux *et al.*, 1990; Rasmussen *et al.*, 1991). However, the mechanism(s) by which various biological inducers of SAR generate these signals and the identity of the actual systemic signal(s) translocated from the induced leaves to distant leaves remain to be determined. Also, it has not been unequivocally shown that cell death is necessary for the induction of SAR.

It is interesting to observe that, although *C. lagenarium* (a necrotizing pathogen of cucumber), Pss61 (an HR-eliciting bacterium on cucumber) and $HrpZ_{Pss}$ (an HR-eliciting protein) all induced SAR in cucumber plants, there were some differences in the induction of PR proteins by these pathogens/protein. While *C. lagenarium*, Pss61 and $HrpZ_{Pss}$ all induced PR-A, PR-B and PR-C in the inoculated leaves, only *C. lagenarium* induced PR-C in systemic leaves to a high level (visible on a PAGE gel). In contrast, PR-B was induced in systemic leaves to high levels only by $HrpZ_{Pss}$ and Pss61. The induction patterns of PR-A, PR-B, PR-C, chitinase, peroxidase and β-glucanase were the same for Pss61 and $HrpZ_{Pss}$, suggesting that $HrpZ_{Pss}$ either is a major inducer of SAR in Pss61 or is representative of SAR inducers produced by Pss61. The differences in the induction of PR proteins by *C. lagenarium* and Pss61/$HrpZ_{Pss}$ may have resulted from different inducers produced by *C. lagenarium* and Pss61/$HrpZ_{Pss}$, respectively. Alternatively, the differences may reflect possible mechan-

istic differences of plant cell death resulting from the HR caused by Pss61 or HrpZ$_{Pss}$ and disease necrosis caused by *C. lagenarium*, respectively, although both types of cell death efficiently trigger SAR in cucumber.

In this study, 80–160 μg ml^{-1} purified HrpZ$_{Pss}$ were used for induction of SAR. HrpZ$_{Pss}$ at these concentrations consistently elicited both HR and SAR in cucumber and tobacco leaves. It is not known whether these concentrations are comparable to the *in vivo* amounts of HrpZ$_{Pss}$ secreted by Pss61. Nor is it known whether the relative activity of purified HrpZ$_{Pss}$ is comparable to that of HrpZ$_{Pss}$ produced by Pss61 *in planta*. Previously, it was shown that Pss61 *hrpZ* mutants carrying transposon-induced mutations in the *hrpZ* gene (complementation group XII) were defective in the elicitation of HR (Huang *et al.*, 1991) and SAR (data not shown). More recently, it was discovered that these transposon-induced *hrpZ* mutations exert a polar effect on five downstream *hrp* genes (*hrpB–F*) in the *hrpZ* operon (Preston *et al.*, 1995; Collmer, personal communication). *hrpB–F*, like *hrpH*, are likely involved in the assembly of the Hrp secretion apparatus (Preston *et al.*, 1995). Therefore, current *hrpZ* mutations affect the expression of not only the *hrpZ* gene but also several other *hrp* genes that are involved in the secretion of HrpZ$_{Pss}$ and, most likely, other HR elicitors/pathogenicity factors. A non-polar *hrpZ* mutant is needed to assess the contribution of HrpZ$_{Pss}$ in the induction of HR and SAR. Recently, several additional proteins traversing the *P. syringae* Hrp secretion pathway have been identified in *P. syringae* pv. *tomato* (Yuan *et al.*, in preparation). It would be interesting to know whether some of these new Hrp-controlled *P. syringae* extracellular proteins can elicit HR and/or SAR.

Although the *hrpH* mutant of Pss61 failed to induce SAR in most experiments, it efficiently induced the accumulation of peroxidase and chitinase in all experiments (Figure 2b and data not shown). The induction of chitinase by *hrp* mutants was also observed by Jakobek and Lindgren (1993). These data suggest that induction of PR proteins is not necessarily a reflection of induction of SAR and that the accumulation of certain PR proteins may not contribute to resistance. In our experiments, only the accumulation of β-glucanase seemed to correlate with the SAR induced by both *C. lagenarium* and Pss61/HrpZ$_{Pss}$ in cucumber. None of the other identified PR proteins were present at high levels in systemic leaves of all cucumber plants that exhibited SAR. Whether β-glucanase is responsible for the resistance of the induced plants to *C. lagenarium*, TNV and *P. s.* pv. *lacrymans* in cucumber remains to be investigated. The relationships between the PR-A, PR-B, and PR-C proteins with β-glucanase, chitinase, or peroxidase are not known.

The demonstration of HrpZ$_{Pss}$ as a proteinaceous inducer of SAR may have important practical implications for plant disease management. Crop plants could be genetically engineered with genes encoding proteinaceous HR/SAR inducers, such as HrpZ$_{Pss}$, under the control of plant promoters inducible by virulent pathogens. If this approach were successful, the HR and SAR would be triggered in otherwise compatible interactions, limiting the disease development.

Experimental procedures

Growth of plants

Cucumber (*Cucumis sativus* L.) plants were grown in plastic pots containing Promix soil. A liquid fertilizer (Peter's 15-16-17, W. R. Grace and Co., Fogelsville, PA), containing 110 p.p.m. nitrogen, was supplied to the water, beginning when the first true leaf was fully open. Plants were grown in a glass greenhouse equipped with high-pressure sodium lights (with a photoperiod of 14 h) to supplement sunlight when necessary.

Preparation of inocula

HrpZ$_{Pss}$ was purified by affinity chromatography from *Escherichia coli* DH5α(pSYH45). pSYH45 is a derivative of pQE30 (Qiagen, Inc.) expressing a hexahistidine–HrpZ$_{Pss}$ (full-length) fusion protein. The first methionine residue of HrpZ$_{Pss}$ was replaced by the following amino acid sequence in the fusion protein: MRGSHHHHHH. The fusion protein was purified according to the manufacturer's instructions. Imidazole (300 mm) was used to elute HrpZ$_{Pss}$ protein, followed by extensive dialysis (3000-fold) in 5 mM $MgCl_2$ at 4°C. The purity of HrpZ$_{Pss}$ fusion protein was estimated by SDS–PAGE analysis to be greater than 95%. The fusion protein at the concentration of 80 μg ml^{-1} elicited a strong HR in tobacco and cucumber leaves, while an identical preparation from DH5α(pQE30) (used as a control in the purification) did not elicit any visible response in the same leaves.

Pseudomonas syringae strains were grown in King's B broth (King *et al.*, 1954) overnight at 30°C. Bacterial suspensions were prepared in 5 mM $MgSO_4$. Spores of *Colletotrichum lagenarium* were prepared as described previously (Kuc and Richmond, 1977). Tobacco necrosis virus inoculum was prepared by grinding cucumber leaves bearing necrotic local lesions in water (1g infected leaf tissue per 10 ml distilled water).

Induction of SAR

First true leaves (Leaf 1) of young cucumber plants (cv. 'Marketer') were treated with test agents by infiltration through their abaxial surfaces at 30 sites per leaf, with 10 μl per site delivered by a repeating pipettor. Treatments consisted of buffer (5 mM $MgSO_4$), HrpZ$_{Pss}$ (final concentration in buffer was 80–160 μg ml^{-1}), Pss61 or *hrpH* (a final OD_{600} = 0.2 in 5 mM $MgSO_4$, equivalent to approximately 2×10^8 cells ml^{-1}), or a spore suspension of *C. lagenarium* (7.5×10^4 spores ml^{-1}).

For experiments involving tobacco (*Nicotiana tabacum* Samsun NN) plants, the third and fourth true leaves of 6-week-old plants were inoculated with TMV (100–150 lesions per leaf) or infiltrated with 120 μg ml^{-1} HrpZ$_{Pss}$ or 5 mM $MgSO_4$. For TMV inoculation, adaxial leaf surfaces were dusted with carborundum and then rubbed with a cheesecloth pad moistened with a TMV suspension. For inoculation with HrpZ$_{Pss}$ or 5 mM $MgSO_4$, 50 μl solution was

pressured into each of 10 panels of a tobacco leaf using a needleless syringe. Five plants were used for each treatment.

Assessment of SAR

At 7–8 days after treatment of Leaf 1 with test agents, subsequently developed leaves (usually Leaf 2 and/or Leaf 3) were challenged with *C. lagenarium*, TNV or *P. s.* pv. *lacrymans*.

For fungal challenge, 20 sites per leaf received 10 µl droplets of a *C. lagenarium* spore suspension (1×10^5 spores ml^{-1}) placed on adaxial surfaces with a repeating pipettor. After inoculation, plants were held in darkened moist chambers for 24 h to facilitate penetration of leaves by the pathogen. Chambers were then gradually opened to allow plant adaptation to ambient conditions over a 12 h period, and plants were then returned to a greenhouse bench for an additional 6–7 days to allow disease development.

For TNV challenge, adaxial leaf surfaces were dusted with carborundum and then rubbed with a cheesecloth pad moistened with a TNV suspension. Virus-inoculated plants were maintained on a greenhouse bench for 8–10 days to permit disease development.

For assessment of SAR to the angular leaf spot bacterium, *P. s.* pv. *lacrymans*, Leaf 1 was infiltrated with buffer, *C. lagenarium*, or $HrpZ_{Pss}$ as described above, and Leaf 5 was challenged on the abaxial surface with the bacterium by spraying with a bacterial suspension ($OD_{600}=0.1$) containing 0.02% Silwet L-77, a surfactant, at 11 days post-induction or by rubbing with a cheesecloth pad saturated with a bacterial suspension ($OD_{600}=0.2$) at 17 days after induction treatment. Spray-inoculated leaves were misted once and plants were then placed in a darkened moist chamber for 18 h, followed by a 12 h acclimation period. Plants were subsequently returned to the greenhouse bench. Rub-inoculated leaves were misted once with water and plants were kept on a greenhouse bench. Disease was allowed to develop for 7 days for rub-inoculated plants or 13 days for spray-inoculated plants.

For evaluation of anthracnose development, the number and diameter of necrotic lesions caused by *C. lagenarium* were determined, and the total necrotic area per leaf was calculated. The extent of disease caused by TNV or *P. s.* pv. *lacrymans* was evaluated by counting necrotic local lesions on entire inoculated leaves.

For assessment of SAR to TMV, the seventh and eighth true leaves were challenge-inoculated with TMV (100–150 lesions per leaf) 5 days after induction. For each treatment the diameters of 100 TMV lesions (from 10 leaves of five plants) were recorded.

PR protein assay

Tissues were collected from Leaf 1 and Leaf 2 during the 14 day period following induction of Leaf 1. The leaf tissues were rapidly frozen with dry ice and stored at –80°C. Protein extraction was based on the method previously described (Ji and Kuc, 1995). Frozen leaf tissues were homogenized at 0–4°C in 0.1 M sodium citrate buffer, pH 5.4, containing 0.1% (v/v) β-mercaptoethanol and 0.1% (w/v) L-ascorbic acid. The homogenate was centrifuged at 12 000 ***g*** for 30 min. The supernatant was decanted and dialyzed against two changes of water for 24 h and then against two changes of 0.05 M sodium acetate buffer (pH 5.0) for 2 h. The extract was centrifuged again at 10 000 ***g*** for 10 min. The supernatant was used as crude enzyme extract. Protein concentrations were measured using the Bio-Rad protein assay kit with bovine gamma globulin as standard.

Determination of enzyme activities in cucumber leaves

Protein patterns and peroxidase isozymes were analyzed after a single separation using a 15% (w/v) native-PAGE gel (Pan *et al.*, 1989). Peroxidase activity was determined using guaiacol as substrate (Hammerschmidt *et al.*, 1982). β-1,3-glucanase and chitinase activities were detected as described elsewhere (Ji and Kuc, 1995).

Expression of pr-1 *gene in tobacco leaves*

An internal fragment (from nt 304 to 535) of the tobacco *pr*-1 gene (Figure 1 in Cornelissen *et al.*, 1986) was amplified in a polymerase chain reaction (PCR) and labeled with [α-^{32}P]-dATP. Total RNA was purified from systemic leaves (the ninth true leaves) of tobacco plants 5 days post-induction. Ten micrograms of RNA from each treatment were fractionated in a 1.2% agarose/formaldehyde gel and subsequently blotted to Immobilon-N membrane (Millipore). Hybridization was performed in a solution consisting of 6×SSC, 2×Denhardt's reagent, 0.1% SDS and 10% dextran sulfate at 55°C. Washes were carried out in 0.2×SSC, 0.1% SDS at 60°C.

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(54) **DISEASE-RESISTANT PLANTS AND METHOD OF CONSTRUCTING THE SAME**

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(57) **ABSTRACT**

It is the object of the present invention to provide disease-resistant plants which have been transformed to cause an effective defense reaction, and methods for producing the same.

The present invention provides expression cassettes comprising a promoter capable of promoting a constitutive, inducible, or organ- or phase-specific gene expression, and a gene, under the control of said promoter, encoding an elicitor protein.

Construct name	Inducible/ Constitutive	Contents of the construct	Plants which the construct was introduced
PALL-hrpZ	Inducible	PAL1.45 pro — *hrpZ*	Tobacco
PALS-hrpZ	Inducible	PAL0.45 pro — *hrpZ*	Tobacco
35S-hrpZ	Constitutive	35S pro — *hrpZ*	Rice, Tobacco
PPDK-hrpZ	Constitutive	PPDK pro — *hrpZ*	Rice, Tobacco

Fig. 1 Constructs introduced into plants

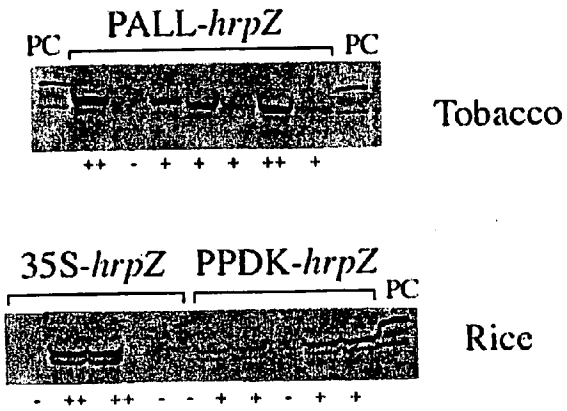
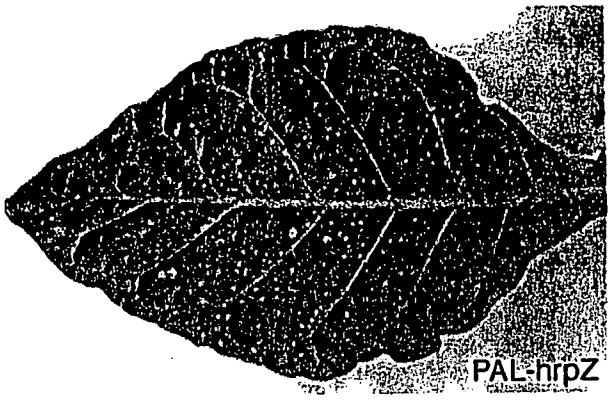
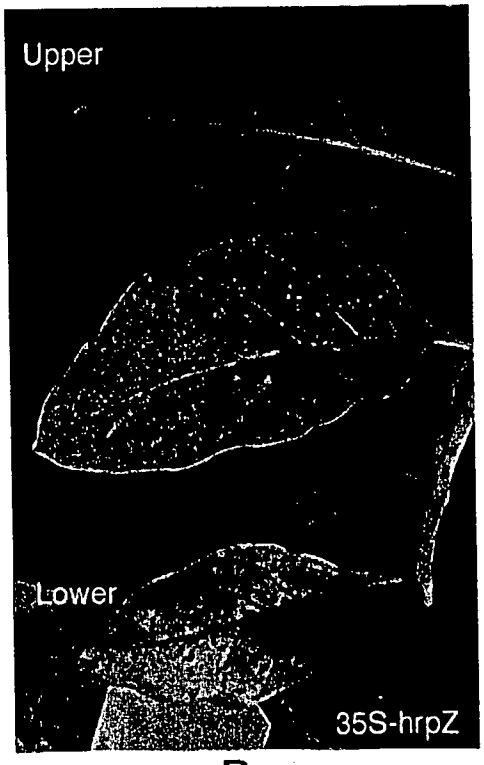


Fig. 2 Expression of harpin$_{pss}$ in tobacco and rice



A



B

Fig. 3 Formation of hypersensitive-response-like localized necrosis spots

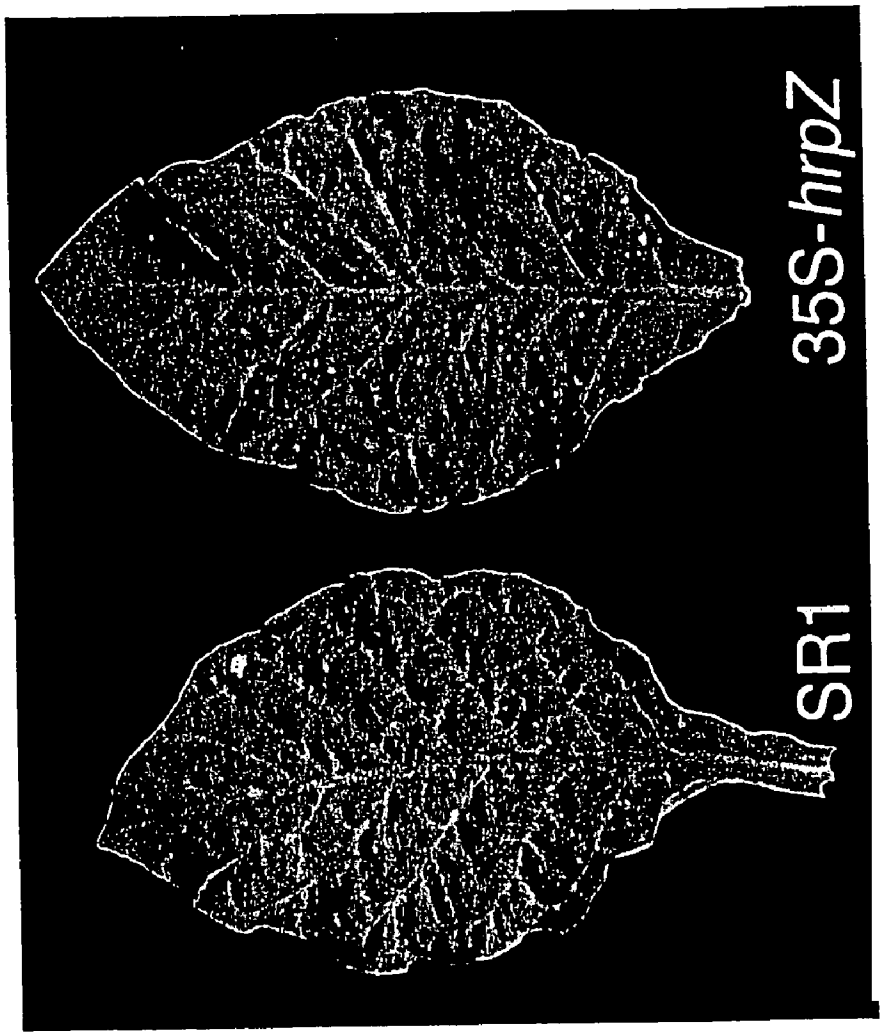


Fig. 4 Resistance to powdery mildew

DISEASE-RESISTANT PLANTS AND METHOD OF CONSTRUCTING THE SAME

FIELD OF THE INVENTION

[0001] The present invention relates to methods for producing disease-resistant plants, gene expression cassettes for producing disease-resistant plants, and transgenic, disease-resistant plants produced by the method.

BACKGROUND OF THE INVENTION

[0002] Plant defense against pathogens differs in its mechanism from that observed in animals. For example, there is known in higher plants a hypersensitive response (HR) mechanism which involves a dynamic resistance reaction to pathogen invasion. When a pathogen invades a plant, plant cells at a site of invasion die in response, whereby pathogens are trapped locally. This reaction is known to be induced as a result of either an incompatible host-pathogen interaction or a non-host-pathogen interaction. Such cell suicide can be understood in terms of a localized, programmed cell death (Dangl et al.: Plant Cell 8: 1973-1807 (1996)). In addition to the mechanism involving HR, other defense reactions, including generation of active oxygen species, reinforcement of a cell wall, production of phytoalexin and biosynthesis of defense-related proteins such as PR proteins, are also known (Hammond-Kosack and Jones: Plant Cell 8: 1773-1791 (1996)). Further, in addition to such localized defense responses, there is known to take place in many cases a defense reaction spreads whereby PR proteins accumulate also in non-infected parts of a plant, whereby resistance is imparted to the entire plant. This mechanism is referred to as systemic acquired resistance (SAR) and continues for several weeks or longer. As a result, the entire plant is made resistant to secondary infection (Sticher et al.: Annu. Rev. Phytopathol. 35: 235-270 (1997)).

[0003] A first reaction of a plant of switching on a highly organized defense reaction such as outlined above is the recognition by the plant of a molecule called an "elicitor" directly or indirectly produced by an invading pathogen. Additionally, complex signal cascades including the subsequent rapid generation of active oxygen species and reversible protein phosphorylation are considered to be important as initial reactions of the defense response (Yang et al.: Genes Dev. 11: 1621-1639 (1997)). There are a wide variety of elicitors, including so-called nonspecific elicitors e.g. oligosaccharides which are products by degradation of cell wall components of many fungi including chitin/chitosan and glucan, or oligogalacturonic acids derived from a plant cell wall, variety-specific elicitors e.g. avirulence gene products of pathogens such as AVR 9 (Avr gene products), and elicitors with an intermediate specificity such as elicitin (Boller: Annu. Rev. Plant Physiol. Plant Mol. Biol. 46: 189-214 (1995)).

[0004] Harpin is a bacterium-derived protein elicitor which induces hypersensitive cell death in a non-host plant (Wei et al.: Science 257: 85-88 (1992), He et al.: Cell 73: 1255-1266 (1993)). Harpin (harpin$_{Ea}$) has been purified as a first bacterium-derived HR-inducing protein from *Erwinia amylovora* Ea321, a pathogen of pear and apple, and *Escherichia coli* transformed with a cosmid containing the hrp gene cluster, and an hrpN gene encoding Harpin has been cloned (Wei et al.: Science 257: 85-88 (1992)). Thereafter, harping$_{pss}$ encoded by hrpZ gene has been identified and characterized from *Pseudomonas syringae* pv. *syringae* 61, a pathogen of a bean, by screening an *Escherichia coli* expression library with an activity of inducing HR to a tobacco leaf as an index (He et al.: Cell 73: 1255-1266 (1993), and Japanese Patent Application Domestic Announcement No. 1996-510127). The homology between these two harpins is low, and a relatively high homology is found only in 22 amino acids. Moreover, the role of a harpin in pathogenicity has not been made clear. In addition to these, as a third protein, PopA protein (which PopA encodes) is identified from *Pseudomonas solanacearum* GMI1000, a pathogen of a tomato, as a protein inducing HR to a non-host tobacco (Arlat et al.: EMBO. J. 13: 543-553 (1994)). Though PopA gene is located on the outside of hrp cluster, differing from hrpN and hrpZ, they are identical in that they are under the control of an hrp regulon. The above three proteins are glycine-rich, heat stable proteins, induce HR to a non-host tobacco and are secreted extracellularly at least in vitro in a manner of depending upon hrp protein. In addition to these are reported HrpW protein from *Pseudomonas syringae* pv. tomato DC3000 as a protein having the same function (Charkowski et al.: J. Bacteriol. 180: 5211-5217 (1998)), hrpZ$_{pst}$ and hrpZ$_{psg}$ proteins as harpin$_{pss}$ homologues (Preston et al.: Mol. Plant-Microbe. Interact. 8: 717-732 (1995)), and harpin$_{Ech}$ (Bauer et al.: Mol. Plant-Microbe. Interact. 8: 484-491 (1995)) and hrpN$_{Ecc}$ protein (Cui et al.: Mol. Plant-Microbe. Interact. 9: 565-573 (1996)) as harpin$_{Ea}$ homologues.

[0005] It has been made apparent from studies upon various metabolic inhibitors that the formation of localized necrosis spots with harpin is not so-called necrosis due to the cytotoxicity of harpin but a cell death resulting from a positive response on the plant side (He et al.: Mol. Plant-Microbe. Interact. 7: 289-292 (1994), and He et al.: Cell 73: 1255-1266 (1993)), and this hypersensitive cell death is thought to be a type of programmed cell death (Desikan et al.: Biochem. J. 330: 115-120 (1998)). The addition of harpin$_{pss}$ into a cell culture of Arabidopsis induces a homologue of gp91-phox, a constituent of NADPH oxidase, which is thought to have an important role in the oxidative burst as an initial reaction of a disease-resistant reaction, (J. Exp. Bot. 49: 1767-1771 (1998)), and mitogen-activated protein (MAP) kinase (Desikan et al.: Planta. 210: 97-103 (1999)). Moreover, a harpin can impart systemic acquired resistance (SAR) to a plant. For example, SAR meditated by salicylic acid and an NIM gene can be induced to an Arabidopsis plant by artificially injecting harpin$_{Ea}$ into the plant cells (Dong et al.: The Plant J. 20: 207-215 (1999)), and Harpin$_{pss}$ can induce SAR to a cucumber and impart a wide spectrum of resistance to fungi, viruses and bacteria (Strobel et al.: Plant J. 9: 431-439 (1996)).

[0006] Thus, there are reports about artificially injecting or spraying purified harpin into a plant and analyzing the induction of a hypersensitive cell death and an acquired resistance reaction (Japanese Patent Application Domestic Announcement No. 1999-506938, Strobel et al.: Plant J. 9: 431-439 (1996), and Dong et al.: The Plant J. 20: 207-215 (1999)). However, there is no report about introducing a gene encoding an elicitor protein such as a harpin into a plant to produce a transgenic plant and analyzing it.

SUMMARY OF THE INVENTION

[0007] It has been anticipated that, when a gene encoding an elicitor protein such as harpin is introduced into a plant, the plant will express an elicitor protein at a certain amount, even in a normal state with no pathogen, or that it will also express an elicitor protein in a certain amount in organs other than those invaded with a disease, and as a result, various unintended reactions occur to prevent the plant from growing normally. The object of the present invention is therefore to provide a disease-resistant transgenic plant which has been transformed to induce a proper defense reaction, and to provide a method for producing the same.

[0008] The present inventors have engaged in studies assiduously, and as a result have found that a transgenic tobacco with hrpZ gene of *Psedomonas syringae* pv. *syringae* LOB2-1 introduced thereinto induces hypersensitive-response-like localized necrosis spots in response to the inoculation of a powdery mildew fungi (*Erysiphe cichoracearum*) to become resistant, which has led to the completion of the present invention. Surprisingly, a plant grew normally when cell-death-inducing harpin was expressed with a constitutive promoter (cauliflower mosaic virus 35S RNA gene promoter) capable of promoting expression in cells of the whole body. In addition, a hypersensitive cell-death-like reaction was induced only after inoculation with a pathogen. Further, the present inventors have found that a transgenic rice with the same hrpZ gene introduced thereinto becomes blast (*Magnaporthe grisea*)-resistant, thus showing the general-applicability of the present invention.

[0009] The present invention provides a transgenic, disease-resistant plant which has been transformed with an expression cassette comprising a promoter capable of promoting a constitutive, inducible, or organ- or phase-specific gene expression and a gene encoding an elicitor protein under the control of said promoter, wherein said plant is capable of effecting the constitutive, inducible, or organ- or phase-specific expression of the elicitor protein in an amount effective for inducing a defense reaction.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] **FIG. 1** shows the constructs constructed and introduced into plants in the present invention.

[0011] **FIG. 2** is a photograph showing exemplary of the detection results using Western analysis for $harpin_{pss}$ accumulation in transgenic tobacco and rice of the T_0 generation. PC represents $harpin_{pss}$ expression in *Escherichia coli* as a control.

[0012] **FIG. 3** is a photograph showing the appearances of localized necrosis spots occurring in a transgenic tobacco of the T_1 generation. A: PALL-hrpZ-introduced individual (5th day after inoculation, harpin expression level: ++), B: 35S-hrpZ-introduced individual (7th day after inoculation, harpin expression level: ++)

[0013] **FIG. 4** is a photograph showing the resistance of a transgenic tobacco of the T_1 generation against powdery mildew. (Right: 35S-hrpZ-introduced individual, harpin expression level: ++, Left: SR1 as a control, 11th day after inoculation in both)

DETAILED DESCRIPTION OF THE INVENTION

[0014] The present invention also provides methods for producing transgenic, disease-resistant plants capable of effecting the constitutive, inducible, or organ- or phase-specific expression of an elicitor protein in an amount effective for inducing a defense reaction. Such methods comprise the steps of: (a) obtaining transgenic plant cells with expression cassettes comprising a promoter capable of promoting a constitutive, inducible, or organ- or phase-specific gene expression and a gene encoding an elicitor protein under the control of said promoter; and (b) regenerating a complete plant from said transgenic plant cell.

[0015] The present invention also provides expression cassettes capable of being employed for producing a transgenic, disease-resistant plants. Such expression cassettes comprise at least: (a) a promoter capable of promoting a constitutive, inducible, or organ- or phase-specific gene expression; and (b) a gene, under the control of said promoter, encoding an elicitor protein. "Elicitor" is a general term used for substances inducing defense reactions in plants, and including heavy metal ions, and cell wall components of pathogens or plants, in addition to proteins. The term "elicitor" as used in the present specification refers to a protein elicitor unless otherwise specified.

[0016] The term "elicitor protein" as used in the present invention can be any protein capable of inducing a proper defense reaction in a plant to be transformed, and preferably a protein possessing a hypersensitive-response-inducing activity against pathogenic microorganisms. It includes harpin and a harpin-like protein having the same function as harpin. "Harpin" is a protein expected to be introduced into a plant in a manner of depending upon hrp gene though the Type III secretion mechanism, and includes, in addition to $harpin_{pss}$, (He et al.: Cell 73: 1255-1266 (1993), and Japanese Patent Application Domestic Announcement[kohyo] No. 510127/96), $harpin_{Ea}$ (Wei et al.: Science 257: 85-88 (1992), and Japanese Patent Application Domestic Announcement[kohyo]No. 506938/99), PopA (Arlat et al.: EMBO. J. 13: 543-553 (1994)), and hrpW protein (Charkowski et al.: J. Bacteriol. 180: 5211-5217 (1998). Additionally the protein possessing a hypersensitive-response-inducing activity can be, for example, (a) a protein consisting of the amino acid sequence of SEQ. ID No. 2; (b) a protein consisting of an amino acid sequence derived from the amino acid sequence of SEQ. ID No. 2 by deletion, substitution, addition or insertion of one or more amino acids, and possessing a hypersensitive-response-inducing activity; or (c) a protein consisting of an amino acid sequence being at least 50% (preferably at least 80%, more preferably at least 90%, and still more preferably at least 97%) homologous to the amino acid sequence of SEQ. ID No. 2, and possessing a hypersensitive-response-inducing activity. A protein consisting of the amino acid of SEQ ID No. 2 is novel. Hence, the present invention provides one of the following proteins: (a) a protein consisting of the amino acid sequence of SEQ. ID No. 2; (b) a protein consisting of an amino acid sequence derived from the amino acid sequence of SEQ. ID No. 2 by deletion, substitution, addition or insertion of one or more amino acids, and possessing a hypersensitive-response-inducing activity; and (c) a protein consisting of an amino acid sequence being at least 97% homologous to the amino acid sequence of SEQ. ID No. 2, and possessing a hypersensitive-response-inducing activity (but known proteins themselves are excluded from the scope of the present invention).

[0017] By "Homology" referred to in connection with amino acid sequences in the present specification is meant a degree of identification of amino acid residues constituting each sequence between sequences to be compared. In homology, the existence of a gap(s) and the nature of an amino acid(s) are taken into consideration (Wilbur, Proc. Natl. Acad. Sci. USA 80: 726-730 (1983) and the like). To calculate homology, commercially available software such as BLAST (Altschul: J. Mol. Biol. 215: 403-410 (1990), and FASTA (Peasron: Methods in Enzymology 183: 63-69 (1990)) can be employed.

[0018] The description "deletion, substitution, addition or insertion of one or more amino acids" as used in the present specification in connection with an amino acid sequence in the means that a certain number of an amino acid(s) are substituted etc. by any well known technical method such as site-specific mutagenesis, or naturally. The number is, for example, up to ten, and is preferably from 3 to up to 5.

[0019] A gene encoding an elicitor protein to be employed in the expression cassette of the present invention can easily be isolated by methods well-known to those skilled in the art.

[0020] The gene encoding an elicitor protein can be, for example, (a) a DNA molecule consisting of the nucleotide sequence of SEQ. ID No. 1; (b) a DNA molecule consisting of a nucleotide sequence derived from the nucleotide sequence of SEQ. ID No. 1 by deletion, substitution, addition or insertion of one or more nucleotides, and encoding a protein possessing a hypersensitive-response-inducing activity; (c) a DNA molecule consisting of a nucleotide sequence being hybridizable with a DNA molecule consisting of the nucleotide sequence complementary to the nucleotide sequence of SEQ. ID No. 1 under stringent conditions, and encoding a protein possessing a hypersensitive-response-inducing activity; or (d) a DNA molecule consisting of a nucleotide sequence being at least 50% (preferably at least 80%, more preferably at least 90%, and still more preferably at least 97%) homologous to the nucleotide sequence of SEQ. ID No. 1, and encoding a protein possessing a hypersensitive-response-inducing activity. A DNA molecule consisting of the nucleotide sequence of SEQ ID No. 1 is novel. Hence, the present invention also provides a gene consisting of one of the following DNA molecules: (a) a DNA molecule consisting of the nucleotide sequence of SEQ. ID No. 1; (b) a DNA molecule consisting of a nucleotide sequence derived from the nucleotide sequence of SEQ. ID No. 1 by deletion, substitution, addition or insertion of one or more nucleotides, and encoding a protein possessing a hypersensitive-response-inducing activity; (c) a DNA molecule consisting of a nucleotide sequence being hybridizable with a DNA molecule consisting of the complementary nucleotide sequence to the nucleotide sequence of SEQ. ID No. 1 under stringent conditions, and encoding a protein possessing a hypersensitive-response-inducing activity; or (d) a DNA molecule consisting of a nucleotide sequence being at least 50% homologous to the nucleotide sequence of SEQ. ID No. 1, and encoding a protein possessing a hypersensitive-response-inducing activity (but known genes themselves such as hrpZ gene of *Pseudomonas syringae* pv. *syringae* 61 are excluded from the scope of the present invention). To calculate homology in connection with nucleotide sequences, commercially available software can be employed.

[0021] By "deletion, substitution, addition or insertion of one or more nucleotides" in connection with a nucleotide sequence in the present specification is meant that a certain number of a nucleotide(s) are substituted etc. by a well-known technical method such as a site-specific mutagenesis or naturally. The number is, for example, up to ten, preferably from 3 to up to 5. By "stringent conditions" referred to in the present specification is meant hybridization conditions wherein the temperature is at about 40° C. or above and that the salt concentration is of about 6×SSC (1×SSC=15 mM sodium citrate buffer; pH: 7.0; 0.15 M sodium chloride; 0.1% SDS), preferably at about 50° C. or above, more preferably at about 65° C. or above.

[0022] The promoter to be employed in the present invention can be any promoter capable of functioning as a promoter for a gene encoding an elicitor protein in a plant to be transformed. In the present invention, a promoter capable of promoting a constitutive, inducible, or organ- or phase-specific gene expression can be employed.

[0023] By "promoter promoting a constitutive gene expression (often referred to as a "constitutive promoter")" is meant a promoter whose organ specificity and/or phase specificity are (is) not high in connection with the transcription of the gene. Examples of the constitutive promoter include cauliflower mosaic virus 35S promoter, ubiquitin promoter (Cornejo et al.: Plant Mol. Biol. 23: 567-581 (1993)), actin promoter (McElroy et al.: Plant Cell 2: 163-171 (1990)), alpha tubulin promoter (Carpenter et al.: Plant Mol. Biol. 21: 937-942 (1993)) and Sc promoter (Schenk et al.: Plant Mol. Biol. 39: 1221-1230 (1999)). In a transgenic plant, the expression cassette promoting the constitutive expression of an elicitor protein includes, for example, a known promoter that is known as a constitutive promoter.

[0024] By "promoter promoting an inducible gene expression (often referred to as an "inducible promoter")" is meant a promoter which induces transcription by physical or chemical stimulation, such as light, disease, injury or contact with an elicitor. Examples of the inducible promoter include pea PAL promoter, Prp1 promoter (Japanese Patent Application No. 1998-500312), hsr203J promoter (Pontier et al.: Plant J. 5: 507-521 (1994)), EAS4 promoter (Yin et al.: Plant Physiol. 115: 437-451 (1997)), PR1b1 promoter (Tornero et al.: Mol. Plant Microbe. Interact. 10: 624-634 (1997)), tap1 promoter (Mohan et al.: Plant Mol. Biol. 22: 475-490 (1993)) and AoPR1 promoter (Warner et al.: Plant J. 3: 191-201 (1993)). In a transgenic plant, the expression cassette promoting an inducible elicitor protein expression includes, for example, a known promoter known as an inducible promoter.

[0025] By "promoter promoting an organ-specific gene expression (often referred to as an "organ-specific promoter")" is meant a promoter giving, to the transcription of the gene, a specificity to an organ, such as a leaf, a root, a stem, a flower, a stamen and a pistil. Examples of the organ-specific promoter include a promoter promoting a high gene expression in green tissues of a photosynthesis-related gene, such as PPDK (Matsuoka et al.: Proc. Natl. Acad. Sci. USA 90: 9586-9590 (1993)), PEPC (Yanagisawa and Izui: J. Biochem. 106: 982-987 (1989) and Matsuoka et al.: Plant J. 6: 311-319 (1994)) and Rubisco (Matsuoka et al.: Plant J. 6: 311-319 (1994)). In a transgenic plant, the

expression cassette promoting an organ-specific elicitor protein expression includes, for example, a known promoter that is known as an organ-specific promoter.

[0026] By "promoter promoting a phase-specific gene expression (often referred to as a "phase-specific promoter")" is meant a promoter giving, to the transcription of the gene, a phase specificity to a phase, such as a initial, middle and later growth phase. Examples of the phase-specific promoter include a promoter functioning specifically in aged leaves such as SAG12 promoter (Gan and Amashino: Science 270: 1986-1988 (1985)).

[0027] Vectors for sub-cloning each DNA fragment as a component of the expression cassette of the present invention can be simply prepared by connecting an intended gene into a vector for recombination (plasmid DNA) available in the art by any common technique. Specific examples of suitable vectors include plasmids derived from *Escherichia coli*, such as pBluescript, pUC18, pUC19 and pBR322, but are not limited only to these plasmids.

[0028] As a vector for introducing the expression cassette of the present invention into a plant to be transformed, a vector for transforming plants can be used. The vectors for plants are not particularly limited, so far as they are capable of expressing the concerned gene and producing the concerned protein in a plant cell, and examples thereof include pBI221, pBI121 (both being manufactured by Clontech) and vectors derived therefrom. In addition, for the transformation of a monocotyledonous plant in particular, there can be exemplified pIG121Hm, pTOK233 (both by Hiei et al.: Plant J. 6: 271-282 (1994)), pSB424 (Komari et al.: Plant J. 10: 165-174 (1996)), superbinary vector pSB21 and vectors derived therefrom. A recombination vector having the expression cassette of the present invention can be constructed by introducing a gene encoding an elicitor protein into any of these known vectors (if required, a promoter region being recombined) by a procedure known well to those skilled in the art. For example, a recombinant vector having an expression cassette comprising a constitutive promoter and hrpZ gene can be constructed by integrating hrpZ gene into superbinary vector pSB21. A recombinant vector having an expression cassette comprising an inducible promoter and hrpZ gene can be constructed by removing the existing promoter from the above recombinant vector and integrating an inducible promoter in place.

[0029] A plant-transforming vector preferably comprises at least a promoter, a translation initiator codon, a desired gene (a DNA sequence of the invention of the present application or a part thereof), a translation termination codon and a terminator. Moreover, it may comprise a DNA molecule encoding a signal peptide, an enhancer sequence, a non-translation region on the 5' side and the 3' side of the desired gene and a selection marker region as appropriate. Examples of marker genes include antibiotic-resistant genes such as tetracyclin, ampicillin, kanamycin or neomycin, hygromycin or spectinomycin; and genes such as luciferase, β-galactosidase, β-glucuronidase(GUS), green fluorescence protein (GFP), β-lactamase and chloramphenicol acetyl transferase (CAT).

[0030] As methods for introducing a gene into a plant can be mentioned a method employing an agrobacterium (Horsch et al.: Science 227: 129 (1985), Hiei et al.: Plant J. 6: 271282 (1994)), a leaf disc method (Horsch et al.: Science 227: 1229-1231 (1985), an electroporation method (Fromm et al.: Nature 319: 791 (1986)), a PEG method (Paszkowski et al.: EMBO. J. 3: 2717 (1984)), a micro-injection method (Crossway et al.: Mol. Gen. Genet. 202: 179 (1986)) and a minute substance collision method (McCabe et al.: Bio/Technology 6: 923 (1988)), but any method for introducing a gene into a desired plant may be employed without any particular limitation. Of these methods for transfection, a method comprising transferring a vector into an agrobacterium by mating and then infecting a plant with the agrobacterium is preferred. Methods for infection is also well-known to those skilled in the art. Examples include a method comprising damaging a plant tissue and infecting it with a bacterium; a method comprising infecting an embryo tissue (including an immature embryo) of a plant with the bacterium; a method comprising infecting with a callus; a method comprising co-culturing protoplasts and the bacterium; and a method comprising culturing a fragment of a leaf tissue together with the bacterium (leaf disc method).

[0031] Successfully transformed cells can be selected from other cells by employing an appropriate marker as an index or examining the expression of a desired trait. The transformed cell can further be differentiated employing a conventional technique to obtain a desired transgenic plant.

[0032] Analysis of the resultant transformant can be performed by employing various methods that are well-known to those skilled in the art. For example, oligonucleotide primers can be synthesized according to the DNA sequence of the introduced gene, and the chromosome DNA of the transgenic plant can be analyzed by PCR employing the primers. In addition, the analysis can be performed on the basis of the existence of mRNA corresponding to the introduced gene and the existence of the protein expression. Moreover, the analysis can be performed on the basis of the appearance of the plant (for example, in the case of transformation with a gene encoding a protein capable of inducing localized necrosis spots, the presence of localized necrosis spots, or the size, number and the like of the localized necrosis spots), disease resistance (for example, the existence of resistance or its degree upon contacting the plant with a pathogen) and the like.

[0033] In the transgenic plant of the present invention, a constitutive, inducible, or organ- or phase-specific expression of an elicitor protein in an amount effective for inducing a defense reaction can be achieved. The amount effective for inducing a defense reaction is such an amount that the expressed elicitor protein can induce at least a localized defense-related reaction (for example, induction of a hypersensitive cell death (localized necrosis)) to the plant. Preferably, the amount is such that the defense reaction extends to the whole body of the plant, and as a result, the whole plant becomes resistant (systemic acquired disease-resistant). Moreover, preferably, the amount is not so large that causes death of the localized tissue having the necrosis spots as a result of the localized necrosis spots becoming too large.

[0034] Moreover, in the transgenic plant of the present invention, an elicitor protein is preferably expressed in an amount which, while being effective for inducing a defense reaction in response to stimulation such as the invasion of a pathogen, does not, under normal conditions, remarkably prevent the growth of the plant due to the negligible or low expression, if any. For example, in the case of employing

$harpin_{pss}$ as an elicitor protein, usually no $harpin_{pss}$ is expressed, or is expressed only in an amount that does not allow localized necrosis spots to cause the death of the organ, and preferably it is expressed in an amount that induces a hypersensitive response at the time of the invasion of a pathogen. Further, it is preferably expressed in such an amount that, even if a pathogen invades to cause $harpin_{pss}$ to accumulate, localized necrosis spots are hardly observable by the naked eye, but the whole body acquires a systemic disease-resistannce.

[0035] In order to induce such a proper defense reaction, for example, a promoter capable of promoting an inducible gene expression is employed. Hence, in one embodiment of the present invention, an inducible promoter and a harpin gene are combined.

[0036] In addition, a proper defense reaction can be accomplished not only in the case of employing an inducible promoter but also in the case of employing a constitutive promoter. Hence, in another embodiment of the present invention, a constitutive promoter and a harpin gene are used in combination. In this embodiment, as a mechanism of the occurrence of a proper defense reaction, it is considered that an elicitor protein, for example, $harpin_{pss}$, is recognized at the outside of cell membranes or on the cell wall of plant cells, and hence, $harpin_{pss}$ accumulating in cytoplasm is not recognized by plant cells until degradation of cells occurs due to invasion of fungus, and as a result, the hypersensitive response appears after the inoculation of the pathogen or it is deduced that there exists a further factor which is related to the inoculation of a pathogen in the mechanism of the occurrence of the elicitor activity of $harpin_{pss}$.

[0037] The transgenic plants of the present invention include a transgenic, powdery mildew-resistant tobacco which has been transformed with an expression cassette comprising a constitutive or inducible promoter and a gene, under the control of said promoter, encoding an elicitor protein such as $harpin_{pss}$, or a transgenic, blast-resistant rice which has been transformed with an expression cassette comprising a constitutive promoter and a gene, under the control of the promoter, encoding an elicitor protein such as $harpin_{pss}$.

[0038] It is thought that the present invention can be applied to plants other than rice and tobacco described in the examples to be described later. Examples of such plants include, as crops, wheat, barley, rye, corn, sugar cane, sorghum, cotton, sunflower, peanut, tomato, potato, sweet potato, pea, soybean, azuki bean, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, onion, carrot, eggplant, pumpkin, cucumber, apple, pear, melon, strawberry and burdock; and, as ornamental plants, arabidopsis thaliana, petunia, chrysanthemum, carnation, saintpaulia and zinnia. The "transgenic plants" referred to in the present invention include not only transgenic plants (To generation) obtained by obtaining a transgenic plant cell according to the method of the present invention and regenerating, from said plant cell, a complete plant, but also later-generation (T_1 generation and the like) plants obtained from said transgenic plants so far as the disease-resistant trait is contained. In addition, the "plants" referred to in the present invention include, unless otherwise spcified, in addition to plants (individuals), seeds (including germinated seeds and immature seeds), organs or parts thereof (including a leaf, a root, a stem, a flower, a stamen, a pistil and pieces thereof), a plant culture cell, a callus and a protoplast.

[0039] The diseases analyzed in the following examples are tobacco powdery mildew and rice blast, but as other diseases of tobacco there can be mentioned wildfire, bacterial wilt and TMV; and as other diseases of rice there can be mentioned sheath blight disease and bacterial leaf blight disease. According to the method for producing a disease-resistant plant of the present invention, it is possible to impart resistance in plants to these diseases.

EXAMPLES

Example 1

Cloning of HrpZ Gene

[0040] A pair of primers for amplifying the open leading frame of hrpZ gene were synthesized in reference to the nucleotide sequence of the reported hrpZ gene of *Pseudomonas syringae* pv. *syringae* 61 (He et al.: Cell 73: 1255-1266 (1993)), and Japanese Patent Application Domestic Announcement[Kohyo] No. 1996-510127):

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Hrp1:  AAA ATC TAG AAT GCA GAG TCT CAG TCT TAA

Hrp2:  AAA AGT CGA CTC AGG CTG CAG CCT GAT TGC
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[0041] Employing these primers, PCR was performed with a DNA molecule of a cosmid clone containing an hrp cluster derived from *Pseudomonas syringae* pv. *syringae* LOB2-1 (a casual agent for bacterial blight of lilac) (Inoue and Takikawa: J. Gen. Plant Pathol. 66: 238-241 (2000)) as a template. PCR was performed under the following conditions: the amount of a reaction solution: 20>1; each primer: 0.5 μM; dNTP: 0.2 mM; 1×ExTaq buffer; ExTaq DNA polymerase (from Takara Shuzo): 1U; once at 95° C. for 5 minutes, then 30 cycles at 94° C. for 30 seconds, at 60° C. for 30 seconds and at 72° C. for 2 minutes, and once at 72° C. for 10 minutes. The PCR product was ligated to a vector pCR2.1 (from Invitrogen) using Takara ligation kit (from Takara Shuzo) and transformed into an *Escherichia coli* TB1 strain. As a result of determining the entire nucleotide sequence of the PCR product, it consisted of 1029 bp in the length, longer than the reported hrpZ gene (He et al.: Cell 73:1255-1266(1993)) by three bases (one amino acid), and showed a homogoly of 96.7% in nucleotides and a homology of 96.5% in amino acids. The reason that the nucleotide sequences are not completely the same is thought to be due to a variation among the pathover. The nucleotide sequence of the cloned hrpZ gene is shown in SEQ. ID No. 1 and the deduced amino acid sequence obtained therefrom is shown in SEQ. ID No. 2, respectively.

Example 2

Expression in an *Escherichia coli* and Production of an Antibody

[0042] The above plasmid with an hrpZ gene integrated into pCR2.1 was digested with restriction enzymes BamHI and SaII, and was subjected to electrophoresis on 0.7% agarose to separate a fragment of about 1.1 kb. This fragment was ligated to an expression vector pQE31 (from QIAGEN) digested with the same enzymes and transformed

into *Eschrichia coli* M15 strain. The thus obtained *Eschrichia coli* was cultured in an LB medium in the presence of 1 mM of IPTG at 37° C., $harpin_{pss}$ was accumulated as insoluble fraction. Since this protein showed poor adsorption to a nickel resin adsorbent, the purification of $harpin_{pss}$ was conducted in the following procedure. The *Eschrichia coli* M15 strain having the pQE31 vector with the hrpZ gene integrated thereinto was cultured in 2 ml of an LB medium containing 100 mg/l of ampicillin and 25 mg/l of kanamycin at 37° C. overnight, and transferred into 250 ml of the LB medium and cultured for about three hours; then 1 mM of IPTG was added thereto and the culture was further conducted at 37° C. for 4 hours. Cells were collected by centrifugation, the insoluble fraction was dissolved in 4 ml of an eluation buffer (8 M urea, 0.1 M sodium dihydrogen phosphate, 0.01 M Tris, pH 8.0), and a supernatant liquid was obtained by centrifugation and subjected to electrophoresis on a 12.5% acrylamide gel containing 0.1% SDS, and then stained with Coomassie Brilliant Blue to cut a band appearing at around 40 kDa. The gel was cut into small pieces, and an elution buffer (1% SDS, 0.02 M Tris HCl, pH of 8.0) was added thereto in an amount ten times the volume of the gel, and shaken for three days. The supernatant was transfered to a dialysis membrane with a cut off molecular weight of 6,000 to 8,000, and the dialysis was conducted with 80% acetone as an external liquid once for 4 hours and once overnight. The whole content in the dialysis tube was moved into an Eppendorf tube, subjected to centrifugation to discard the supernatant, and the pellet was dried to obtain a purified $harpin_{pss}$ preparation. 3 mg of the purified $harpin_{pss}$ was sent to Sawady Technology for the production of an antibody (anti-rabbit $harpin_{pss}$ serum).

Example 3

Construction of a Gene and Transformation of a Plant

[**0043**] The hrpZ gene integrated into pCR2.1 was excised from the vector by digestion with restriction enzymes XbaI and SacI (from Takara Shuzo). On the other hand, superbinary vector pSB21 (35S-GUS-NOS, Komari et al.: Plant J. 10: 165174 (1996)) was digested with the same enzymes to remove the GUS gene, and the hrpZ gene was integrated thereinto. According to the above procedure, a construct named 35S-hrpZ (35S promoter-hrpZ gene-NOS terminator) was constructed. The cauliflower mosaic virus 35S promoter is a promoter capable of constitutively promoting a high expression, and it is anticipated that rice and tobacco transformed with this construct will accumulate $harpin_{pss}$, the hrpZ gene product, in the whole body.

[**0044**] pSB21 was digested with restriction enzymes HindIII and XbaI to remove the 35S promoter, and a 0.9 kb fragment of corn PPDK promoter (Taniguchi et al.: Plant Cell Physiol. 41: 42-48 (2000)) was integrated thereinto. The resulting plasmid was digested with XbaI and SacI to remove the GUS gene, and then the above-described hrpZ XbaI-SacI fragment was inserted thereinto. Thus, PPDK-hrpZ (PPDK promoter-hrpZ gene-NOS terminator) was constructed. The corn PPDK promoter is a promoter capable of promoting a strong expression in photosynthesis organs such as mesophyl cells (Taniguchi et al.: Plant Cell Physiol. 41: 42-48 (2000)), and it is anticipated that rice plants transformed with this construct will accumulate $harpin_{pss}$, the hrpZ gene product, in green organs (leaves).

[**0045**] PAL promoter was cloned as below. Plasmid DNA was extracted from agrobacterium LBA4404 strain (gifted from Prof. Shiraishi of Okayama University) having a construct containing PSPAL1 (PSPAL1 promoter-GUS gene-NOS terminator) (Yamada et al.: Plant Cell Physiol. 35: 917-926 (1994), and Kawamata et al.: Plant Cell Physiol. 38: 792-803 (1997)). On the other hand, a reverse primer and two forward primers were designed on the basis of the nucleotide sequence of the reported PSPAL1 promoter (Patent: JP 1993153978-A 1 22-Jun.-1993; TAKASAGO INTERNATL. CORP.):

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PALRVXba:
GGG GTC TAG AAT TGA TAC TAA AGT AAC TAA TG

PALFFHin:
TTG GAA GCT TAG AGA TCA TTA CGA AAT TAA GG

PALFSHin:
CTA AAA GCT TGG TCA TGC ATG GTT GCT TC
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[**0046**] A promoter region (PAL-S) of about 0.45 kb in the upstream of the starting point of translation (about 0.35 kb at the upstream of the initiation point of transcription) was amplified by the combination of PALRVXba and PALFSHin, and a promoter region (PAL-L) of about 1.5 kb by the combination of PALRVXba and PALFFHin. The above-mentioned agrobacteruium plasmid DNA was used as a template and PCR was conducted with these primers. The reaction conditions of PCR were as below: reaction solution: 50 μl; each primer: 0.5 μM, dNTP: 0.2 mM; 1×ExTAq buffer, ExTAq DNA polymerase (from Takara Shuzo): 1U; and the reaction was conducted once at 94° C. for three minutes, then 30 cycles at 94° C. for one minute, at 50° C. for one minute and at 72° C. for two minutes, and once at 72° C. for 6 minutes. A PCR product was cloned to vector pCR11 (from Invitrogen).

[**0047**] Since the PsPAL1 promoter had a HinIII site at the upstream 142 bp from the starting point of translation, PAL-S was digested completely with restriction enzyme XbaI and then partially with HindIII to obtain a 0.45 kb of fragment from pCR11. The above mentioned pSB21 was digested with HindIII and XbaI to remove the 35S promoter, and PAL-S was integrated thereinto. In the pSB21 vector employed here the unique PvuII site existing in the basic structure had been removed, and, instead, a PvuII linker had been placed at the unique ECOR1 site (just after the Nos terminator). The plasmid with PAL-S integrated thereinto was further digested with XbaI and SacI to remove the GUS gene, and then the above mentioned 1.1 kb hrpZ XbaI-SacI1 fragment was inserted therein. PALS-hrpZ was constructed according to the above procedure. Next, PAL-L integrated into pCR11 was digested with restriction enzymes XhoI and XbaI to take out a 1.45 kb PAL promoter, which was integrated into vector pSB11 (Komari et al.: Plant J. 10: 165-174 (1996)) co-digested with the same enzymes. The formed plasmid was digested with XbaI and SmaI, and an XbaI-PvuII fragment of PALS-hrpZ (hrpZ-NOS terminator) was inserted therein. In this manner, PALL-hrpZ was produced. The PAL promoter promotes a low-level expression constitutively, but it is a promoter strongly induced with a pathogen and an injury (Yamada et al.: Plant Cell Physiol. 35: 917-926 (1994), and Kawamata et al.: Plant Cell Physiol. 38: 792-803 (1997)), and it is anticipated that a tobacco plant transformed with PALS-hrpZ or PALL-hrpZ accumulates

more harpin$_{pss}$ at the place of stress when these stresses occur. In this case, it is anticipated that more harpin$_{pss}$ will accumulate in the case of PALL relative to the case of PALS.

[0048] According to the tri-parental mating system, of *Escherichia coli* LB392 strain containing the thus produced four constructs 35S-hrpZ, PALS-hrpZ, PALS-hrpZ and PALL-hrpZ (summarized in **FIG. 1**), agrobacterium LBA4404 strain containing a vector pSB4U with a selection marker gene integrated thereinto (corn ubiquitin promoter-hygromycin-resistant gene (hptII)-NOS terminator) and *Escherichia coli* HB101 containing a helper plasmid pRK2013, the hrpZ gene containing construct was introduced into an agrobacterium utilizing homologous recombination.

[0049] The transformation of a tobacco was performed by the leaf disc method (Horsch et al.: Science 227: 1229-1231 (1985)). A leaf of tobacco variety SR1 grown in a greenhouse was sterilized by treatment with ethanol for 30 seconds and with antiformin diluted 5 times for 5 minutes, and after it was cleaned with sterilized water twice, it was cut into one-centimeter squares, and an agrobacterium suspension was inoculated thereto. The concentrations of hygromycin at the time of induction and selection of a transfected shoot and at the time of rooting were 50 or 100 mg/ml and 0 or 50 mg/ml, respectively. For the transformation of rice, immature-embryo-derived cali of varieties of paddy rice, Tsukinohikari, and Koshihikari were transformed employing agrobacterium according to the method of Hiei et al.: Plant J. 6: 271-282 (1994).

Example 4

Analysis of Transformants

[0050] (1) Transgenic Tobacco

[0051] 15 individuals of the re-generated plant were obtained from 35S-hrpZ, 10 individuals were from PALS-hrpZ and 16 individuals were from PALL-hrpZ. There was observed no remarkable difference between the constructs in transformation efficiency. Western analysis was performed on the primary generation (To) of the transformant, and Western analysis and disease assays were performed on the self-pollinated next generation (T_1).

[0052] 1) Western Analysis of T_0 Generation

[0053] 2×2 cm of a leaf of a transgenic tobacco of the 4 or 5 leaf stage and 2×2 cm of a leaf of a non-transgenic tobaco (SR1) were pulverized in 0.1 M HEPES-KOH pH 7.6 buffer in a mortar. The supernatant liquid after centrifugation with 15000 g for 10 minutes was made a protein sample. The amount of the protein was determined with a Bio-Rad Protein Assay kit (from BIO-RAD). About 20 μg of the protein was fractioned by the SDS-PAGE method according to the method of Laemmni et al. (Nature 227: 680-685 (1970)), on 12.5% PAGEL (from ATTO). After electrophoresis, the protein bands on the gel were transferred to a PVDF membrane (from Millipore). The PVDF membrane was placed in a 1×TBS buffer containing 0.5% skim milk for 30 minutes, and shaken in the same buffer containing 1/1000 (v/v) of anti-harpin$_{pss}$ serum at room temperature overnight. As a secondary antibody was employed an anti-goat rabbit IgG peroxidase labeled conjugate (from MBL) or an anti-goat rabbit IgG alkaline phosphatase conjugate (from BIO-RAD) at the concentration of 1/1000 (v/v). As color development systems were employed HRP Color Development Reagent (from BIO-RAD), alkaline phosphatase substrate kit II (from Vector Laboratories). The amounts of the protein expressed were calculated by comparison with the color development of the harpin$_{pss}$ sample of a known concentration, by using a densitometer (model GS-670, from BIO-RAD). Some of the results of the Western analysis of the T_0 generation is shown in **FIG. 2**, and the whole results are summarized in Table 1.

[0054] The expression level is shown in four stages (+++, ++, +, −), which show 0.1% or more of the total soluble proteins (+++), 0.05 to 0.1% (++), 0.05% or less (+) and below the detection limitation (−) in the amount of expression, respectively. This is true also in Tables 2, 3 and 4 to be described later.

TABLE 1

Results of the Western Analysis of the Tobacco T_0 Generation

Construct	Number of re-generated individuals	Expression level of Harpin$_{pss}$[a]			
		−	+	++	+++[b]
PALS-hrpZ	10	1	8	1	0
PALL-hrpZ	16	2	10	4	0
35S-hrpZ	15	6	2	1	6
SR1		3	0	0	0

[a]Each numerical value shows the number of individuals showing each expression level.
[b]The expression level of harpin$_{pss}$ is shown in four stages (+++: particularly high expression, ++: high expression, +: moderate to poor expression, −: below the detection limitation).

[0055] In the case of the constructs having a PAL promoter, the accumulation of harpin$_{pss}$ was detected in 80% or more of individuals. As anticipated, PALL had a larger proportion of high-expression individuals (++) than PALS. On the other hand, in the case of the construct having a 35S promoter, though no accumulation of harpin$_{pss}$ was detected in 6 individuals of the 15 individuals, high-expression individuals were obtained in 7 individuals, near half of the total individuals. Besides, a very high expression (+++) was shown in 6 individuals. Interestingly, no morphological change was observed in the organ of any of a leaf, a stem, a root or a flower of these high-expression individuals, and seed fertility was normal in almost all of them.

[0056] 2) Western Analysis of the T_1 Generation and Disease Resistance Assay

[0057] Reaction to powdery mildew fungus (*Erysiphe cichoracearum*) was analized in about 8 lines of KH1-2 (PALS-hrpZ), KC6-7 (PALL-hrpZ), KC8-1 (PALL-hrpZ), KK1-1 (35S-hrpZ), KK3-8 (35S-hrpZ), KK4-2 (35S-hrpZ), KK4-3 (35ShrpZ), KK7-6 (35S-hrpZ), in which the amount of harpin$_{pss}$ accumulated was high in the T_0 generation.

[0058] Tobacco individuals in which harpin$_{pss}$ was accumulated at a high level in the T_0 generation were selected, and seeds of self-pollinated next generation (T_1) thereof were obtained. The seeds were sowed and observed for about two months, but no visual morphological change was observed for this period; they grew normally in the same manner as the To generation, and no hypersensitive response was observed on the surface of a leaf. Then, powdery

mildew fungi were sprayed to inoculate upon the T_1 generation of the transgenic tobacco of the 4 or 5 leaf stage and a disease resistance assay was performed. About 2 L of a suspension of powdery mildew fungi spores (1.4×106 spores/ml) was spray-inoculated to 244 recombinants and 41 original individuals. As a result, hypersensitive-response-like localized necrosis spots were induced onto a lower leaf of the recombinant 4 or 5 days after inoculation (**FIG. 3A, B**). Surprisingly, not only in the case of the PAL-hrpZ constructs but also in the case of the 35S-hrpZ constructs employing a constitutive promoter, specific localized necrosis spots were induced after the pathogen infection (**FIG. 3B**). The expression frequency of localized necrosis spots on the 5th day after the inoculation was about 5% in the non-transformants, but the frequency was from 6 to 14 times grater in the 35S-hrpZ construct (30 to 71%), from 4 to 5 times greater in the PAL-hrpZ constructs (20 to 27%) (Table 2), and thereafter, in the case of the PAL-hrpZ constructs, the number of local necrosis spots gradually increased. This was assumed to be due to the response of the PsPAL1 promoter to *Erysiphe cichoracearum*. Though the amount of $harpin_{pss}$ accumulated and the degree of the formation of localized necrosis spots tended to be positively correlative (Table 3), there were some exceptional transformants in which no accumulation of $harpin_{pss}$ was detected at least in our Western analysis but localized necrosis spots occurred.

[**0059**] Next, in order to examine whether the localized necrosis spots having occurred after the powdery mildew infection were related to disease resistance, the symptom of powdery mildew on the 11th day after the inoculation thereof was examined. As a result, while there existed no individual in which the spread of powdery mildew hyphae was prevented in the non-transformants, from 15 to 57% individuals in the case of 35S-hrpZ constructs and from 13 to 18% individuals in the case of PAL-hrpZ constructs showed apparently less significant symptom as compared to the non-transformants (**FIG. 4**, Table 2). The prevenstion of that the spread of powdery mildew was observed not only in leaves with localized necrosis spots but also in middle or upper leaves with no localized necrosis spots, and this is thought to be due to systemic acquired resistance (SAR). As a result of observing the hyphae of powdery mildew by cotton blue dyeing, the hyphae of powdery mildew extended sharply and spread around the surface in infested leaves of the SR1 of the original line as a control, whereas, though haustorium is formed on the surface of a leaf in the transformants, the spreading of hyphae was prevented and stopped halfway. The promoters employed in the present studies are 35S promoter (constitutive) and PAL promoter (inducible); and it was found that when 35S promoter was employed instead of PAL promoter, the frequency of localized necrosis spots was higher, and it was further found that at least according to examination on the 11th day after inoculation, more individuals with a strong disease resistance were obtained (Table 2). However, it was observed that, in the case of employing the 35S promoter, the localized necrosis spots formed in response to the pathogen became larger (occupying 10% or more of the leaf area) in some individuals, and as a result, lower leaves died out. In addition, inversely, in some individuals with $harpin_{pss}$ accumulated therein, localized necrosis spots were not observable by the naked eye (Table 2), but some of such individuals had resistance to powdery mildew (of individuals with – of localized necrosis spots in Table 2, individuals of the number in parentheses; the amount of $harpin_{pss}$ expressed is ++ in all). This is thought to be probably due to the occurrence of a hypersensitive response in very small range, and it is possible that a disease-resistant plant with a high practicability can be obtained by the selection of such individuals. According to the fact that no localized necrosis spot occurred without the invasion of the pathogen even in the case where the transription of hrpZ gene was controlled with a constitutive promoter, it is possible to deduce that, since $harpin_{pss}$ was recognized on the outside of a transmembrane or cell wall of plant cells, probably $harpin_{pss}$ accumulated in cytoplasm was not recognized for plant cells till the degradation of cells due to the invasion of the fungi, and as a result, it caused a hypersensitive response after the inoculation of the pathogen. Another possibility may be that the elicitor activity of $harpin_{pss}$ requires the existence of some other factors derived from the pathogen or the plant, induced by the inoculation of the pathogen.

TABLE 2

Relationship among the Amount of $harpin_{pss}$ Accumulated, the Formation of Localized Necrosis Spots and Disease Resistance of the Tobacco T_1 Generation

Line Name	Construct	Expression level (T_0)	Number of individuals analyzed (T_1)
KH1-2	PALS-hrpZ	++	18
KC6-7	PALL-hrpZ	++	43
KC8-1	PALL-hrpZ	++	44
KK1-1	35S-hrpZ	+++	23
KK3-8	35S-hrpZ	+++	33
KK4-2	35S-hrpZ	++	35
KK4-3	35S-hrpZ	+++	7
KK7-6	35S-hrpZ	+++	41
SR1	(control)	–	41

TABLE 2-continued

Relationship among the Amount of harpin$_{pss}$ Accumulated, the Formation of Localized Necrosis Spots and Disease Resistance of the Tobacco T_1 Generation

Line Name	Number of individuals with localized necrosis spots (Number of individuals with less progress of disease spots) +++	++	+	–[a]	Rate of individuals with localized necrosis spots (5th day after inoculation)	Rate of individuals with less progress of disease spots (11th day after inoculation)
KH1-2(PALS)	0	0	5(3)	13(0)	27%	16%
KC6-7(PALL)	0	1(1)	8(6)	34(1)	20%	18%
KC8-1(PALL)	0	1(0)	11(5)	32(1)	27%	13%
KK1-1(35S)	0	0	7(3)	16(1)	30%	17%
KK3-8(35S)	0	2(0)	11(5)	20(0)	39%	15%
KK4-2(35S)	1(1)	4(3)	15(6)	15(0)	57%	28%
KK4-3(35S)	0	3(3)	2(1)	2(0)	71%	57%
KK7-6(35S)	1(1)	4(4)	18(4)	18(1)	56%	24%
SR1 (control)	0	0	2(0)	39(0)	5%	0%

[a]The degree of localized necrosis spots is shown in four stages (+++: very high, ++: high, +: low, –: nil).

[0060]

TABLE 3

Relationship between the Expression level of Harpin$_{pss}$ and the Number of Localized Necrosis Spots in the Tobacco T_1 Generation

Expression level of harpin$_{pss}$[a] (Western analysis)	Degree of localized necrosis spots[b] +++	++	+	–	Incidence of localized necrosis spots
+++	1	4	19	19	56%
++	0	5	32	77	32%
+	1	6	18	38	40%
–	0	1	5	18	25%
SR1	0	0	2	39	5%

[a]The expression level of harpin$_{pss}$ is shown in four stages (+++: particularly high expression, ++: high expression, +: moderate to poor expression, –: below the detection limit) (SR1, –).
[b]The degree of localized necrosis spots is shown in four stages (+++: great many, ++: many, +: few, –: nil).

[0061] (2) Transgenic Rice

[0062] 1) Western Analysis of the T_0 Generation

[0063] Harpin$_{pss}$ was introduced into a rice variety, Tsukinohikari. 35 individuals of the regenerated plant were obtained from the 35S-hrpZ construct, and 26 individuals of the regenerated plant were obtained from the PPDK-hrpZ construct. There was observed no remarkable difference between the constructs in transformation efficiency. Western analysis was performed on the primary generation (T_0) of the transformation and individuals with a high expression were selected.

[0064] Protein was extracted from the regenerated transgenic rice (Tsukinohikari) in the same manner as in the example of the tobacco and subjected to Western analysis. The results of Western analysis of the T_0 generation are shown in Table 4.

TABLE 4

Results of the Western Analysis of the T_0 Generation of Rice (Tsukinohikari)

Construct	Number of regenerated individuals	Expression level of harpin$_{pss}$[a] –	+	++	+++[b]
35S-hrpZ	35	17	5	13	0
PPDK-hrpZ	26	9	13	4	0

[a]Each numerical value shows the number of individuals showing each expression level.
[b]The Expression level of harpin$_{pss}$ is shown in four stages (+++: particularly high expression, ++: high expression, +: moderate to poor expression, –: below the detection limit).

[0065] In the case of the rice (Tsukinohikari), similar to the case of the tobacco, individuals with a high-expression of harpin$_{pss}$ were obtained (see also **FIG. 2**). In the case of a construct having a 35S promoter, the accumulation of harpin$_{pss}$ was detected in about half of the individuals, and the rate of high-expression individuals (++) was about one-third or more of the whole. Also, in the case of a PPDK promoter the accumulation of harpin$_{pss}$ was detected in about two-thirds of the individuals, and of them, 4 individuals showed a high expression. Interestingly, no morphological change was observed in the organ of any of a leaf, a root or a flower of these high-expression individuals. And seed fertility was normal in almost all of them, and T_1 seeds of high-expression individuals could be obtained.

[0066] 2) Western Analysis of the T_0 Generation and the Disease Resistance Assay of the T_1 Generation

[0067] Next, harpin$_{pss}$ was introduced into Koshihikari, one of the most important varieties of rice of Japan. The results of the Western analysis of the T_0 generation are shown in Table 5.

TABLE 5

Results of the Western Analysis of the T_0 Generation of Rice (Koshihikari)

Construct	Number of regenerated individuals	Expression level of harpin$_{pss}$[a] –	+	++	+++[b]
35S-hrpZ	78	18	33	21	6
PPDK-hrpZ	27	7	13	7	0

[a]Each numerical value shows the number of individuals showing each expression level.
[b]The expression level of harpin$_{pss}$ is shown in four stages (+++: amount of accumulation of 0.5% or more to the total soluble leaf proteins, ++: amount of accumulation of from 0.1 to 0.5%, +: amount of accumulation of from 0.01 to 0.1%, –: below the detection limit).

[0068] Of the individuals of the T_0 generation with the 35ShrpZ construct introduced thereinto, four individuals showning a large amount (+++ in Table 5) of the accumulation of harpin$_{pss}$ (hrp5-8, hrp23-5, hrp24-1, hrp429) were selected, and their vulnerability to rice blast in the T_1 generation was examined. The seed fertility of the selected four high-expression individuals was normal, and many self-fertilized seeds could be obtained. T_1 seeds were sowed in a seedling case with culture soil in a manner of 8 seeds×2 rows, cultivated in a greenhouse, and subjected to a disease assay at the 4.8 to 5.2 leaf stage. As a rice blast fungus (*Magneporthe grisea*) was employed race 007. For inoculation, a conidium formed by culturing the blast fungi on an oatmeal sucrose agar medium at 28° C. under dark condition and then, after the spread of the fungi, at 25° C., irradiating near ultraviolet light for three days was employed. The inoculation of the blast fungi was performed by spray-inoculating 30 ml of a suspension adjusted to 1.5×10^5 condia/ml in 0.02% Tween 20 per three seedling cases. The spray-inoculated rice was held in a moistening incubator (SLPH-550-RDS, manufactured by Nippon Medical & Chemical Instruments Co. Ltd.) for 24 hours after the inoculation at 25° C. at a humidity of 100%, and then transferred into the greenhouse. The conditions of the greenhouse were set at 25° C. under light conditions for 16 hours, and at 22° C. under dark conditions for 8 hours. The evaluation of disease resistance was performed by visually counting the number of progressive disease spots on the 5th leaf at 6th day after the inoculation, said leaf being the topmost development leaf at the time of inoculation. Significant differences among the results were evaluated according to the Mann-Whitney U test.

[0069] As a result, though no localized necrosis spot due to the inoculation of the blast fungi was observed, the average number of progressive disease spots was reduced by 24 to 38% relative to the control Koshihikari in three lines (hrp5-8, hrp42-9, hrp23-5) out of the four lines of the harpin$_{pss}$-introduced rice. Moreover, this reduction was statistically significant (Table 6). The above results show that the disease resistance of rice could be increased by the introduction of harpin$_{pss}$.

TABLE 6

Results of the Disease Test against Rice Blast of the Four Lines of Harpin$_{pss}$-Intorduced Rice (T_1 Generation)

Strain	Number of tested individuals	Number of average progressive disease spots[a] (standard error)	Significant Test[b]
hrp5-8	16	9.3 (±1.0)	significant (significance level 1%)
hrp23-5	21	11.4 (±1.3)	significant (significance level 5%)
hrp24-1	20	14.4 (±1.4)	No significant difference
hrp42-9	14	9.4 (±1.4)	significant (significance level 1%)
Koshihikari	64	15.0 (±0.7)	—

[a]Results of the 5th leaf on the 6th day after inoculation
Significant difference to Koshihikari in the Mann-Whitney U test

[0070] As a result of the present invention, it has become apparent for the first time that disease resistance can be imparted to a plant by connecting a gene enconding harpin to a constitutive promoter or an inducible promoter and introducing the gene into the plant. This harpinin-introduced plant is thought to be useful for explicating the function of harpin as a protein elicitor, and also for explicating the mechanism of localized or systemic acquired resistance. In addition, it is revealed that the production of a harpin-introduced resistant plant, which has been thought to be difficult without the use of an inducible promoter, can sufficiently be realized by employing a constitutive promoter, and the extension of the application range of the present approach can be shown. The present invention shows that a method for producing a disease-resistant plant by integrating a DNA sequence encoding a harpin into an expression cassette comprising a sequence of an appropriate constitutive, or organ- or phase-specific promoter capable of functioning in a plant cell, or a promoter induced with stress or pests, and a sequence of a terminator capable of functioning in a plant cell, and introducing it into the plant cell to obtain a regenerated individual is a useful and effective approach in view of genetic engineering.

SEQUENCE LISTING

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1               5                   10                  15

gcc ctt gtc ctg gta cgt cct gaa acc gag acg act ggc gcc agt acg       96
Ala Leu Val Leu Val Arg Pro Glu Thr Glu Thr Thr Gly Ala Ser Thr
            20                  25                  30

tcg agc aag gcg ctt cag gaa gtt gtc gtg aag ctg gcc gag gaa ctg      144
Ser Ser Lys Ala Leu Gln Glu Val Val Val Lys Leu Ala Glu Glu Leu
        35                  40                  45

atg cgc aat ggt caa ctc gac gac agc tcg cca ttg ggc aaa ctg ctg      192
Met Arg Asn Gly Gln Leu Asp Asp Ser Ser Pro Leu Gly Lys Leu Leu
    50                  55                  60

gcc aag tcg atg gcc gcg gat ggc aag gca ggc ggc ggt atc gag gat      240
Ala Lys Ser Met Ala Ala Asp Gly Lys Ala Gly Gly Gly Ile Glu Asp
65                  70                  75                  80

gtc atc gct gcg ctg gac aag ctg att cat gaa aag ctg ggt gac aac      288
Val Ile Ala Ala Leu Asp Lys Leu Ile His Glu Lys Leu Gly Asp Asn
                85                  90                  95

ttc ggc gcg tct gcg gac aac gcc tcg ggt acc gga cag cag gac ctg      336
Phe Gly Ala Ser Ala Asp Asn Ala Ser Gly Thr Gly Gln Gln Asp Leu
            100                 105                 110

atg act cag gtg ctc agt ggc ctg gcc aag tct atg ctc gat gat ctt      384
Met Thr Gln Val Leu Ser Gly Leu Ala Lys Ser Met Leu Asp Asp Leu
        115                 120                 125

ctg acc aag cag gat ggc ggg gca agc ttc tcc gaa gac gat atg ccg      432
Leu Thr Lys Gln Asp Gly Gly Ala Ser Phe Ser Glu Asp Asp Met Pro
    130                 135                 140

atg ctg aac aag atc gcg cag ttc atg gat gac aat ccc gca cag ttt      480
Met Leu Asn Lys Ile Ala Gln Phe Met Asp Asp Asn Pro Ala Gln Phe
145                 150                 155                 160

ccc aag ccg gac tcg ggt tcc tgg gtg aac gaa ctc aag gaa gac aac      528
Pro Lys Pro Asp Ser Gly Ser Trp Val Asn Glu Leu Lys Glu Asp Asn
                165                 170                 175

ttc ctt gat ggc gac gaa acg gct gcg ttc cgc tcg gca ctc gac atc      576
Phe Leu Asp Gly Asp Glu Thr Ala Ala Phe Arg Ser Ala Leu Asp Ile
            180                 185                 190

att ggc cag caa ctg ggt aat cag cag agt ggc gct ggc ggt ctg gcg      624
Ile Gly Gln Gln Leu Gly Asn Gln Gln Ser Gly Ala Gly Gly Leu Ala
        195                 200                 205

ggg acg ggt gga ggt ctg ggc act ccg agc agt ttt tct aac aac tcg      672
Gly Thr Gly Gly Gly Leu Gly Thr Pro Ser Ser Phe Ser Asn Asn Ser
    210                 215                 220

tcc gtg acg ggt gat ccg ctg atc gac gcc aat acc ggt ccc ggt gac      720
Ser Val Thr Gly Asp Pro Leu Ile Asp Ala Asn Thr Gly Pro Gly Asp
225                 230                 235                 240

agc ggc aat agc agt ggt gag gcg ggg caa ctg atc ggc gag ctt atc      768
Ser Gly Asn Ser Ser Gly Glu Ala Gly Gln Leu Ile Gly Glu Leu Ile
                245                 250                 255
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gac cgt ggc ctg caa tcg gta ttg gcc ggt ggt gga ctg ggc aca ccc      816
Asp Arg Gly Leu Gln Ser Val Leu Ala Gly Gly Gly Leu Gly Thr Pro
            260                 265                 270

gta aac acc ccg cag acc ggt acg gcg gcg aat ggc gga cag tcc gct      864
Val Asn Thr Pro Gln Thr Gly Thr Ala Ala Asn Gly Gly Gln Ser Ala
        275                 280                 285

cag gat ctt gac cag ttg ctg ggc ggc ttg ctg ctc aag ggc ctt gaa      912
Gln Asp Leu Asp Gln Leu Leu Gly Gly Leu Leu Leu Lys Gly Leu Glu
    290                 295                 300

gcg acg ctc aag gat gcc ggt caa acc gct acc gac gtg cag tcg agc      960
Ala Thr Leu Lys Asp Ala Gly Gln Thr Ala Thr Asp Val Gln Ser Ser
305                 310                 315                 320

gct gcg caa atc gcc acc ttg ctg gtc agt acg ctg ctg caa ggc acc     1008
Ala Ala Gln Ile Ala Thr Leu Leu Val Ser Thr Leu Leu Gln Gly Thr
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cgc aat cag gct gca gcc tga                                        1029
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Ser Ser Lys Ala Leu Gln Glu Val Val Val Lys Leu Ala Glu Glu Leu
        35                  40                  45

Met Arg Asn Gly Gln Leu Asp Asp Ser Ser Pro Leu Gly Lys Leu Leu
    50                  55                  60

Ala Lys Ser Met Ala Ala Asp Gly Lys Ala Gly Gly Gly Ile Glu Asp
65                  70                  75                  80

Val Ile Ala Ala Leu Asp Lys Leu Ile His Glu Lys Leu Gly Asp Asn
                85                  90                  95

Phe Gly Ala Ser Ala Asp Asn Ala Ser Gly Thr Gly Gln Gln Asp Leu
            100                 105                 110

Met Thr Gln Val Leu Ser Gly Leu Ala Lys Ser Met Leu Asp Asp Leu
        115                 120                 125

Leu Thr Lys Gln Asp Gly Gly Ala Ser Phe Ser Glu Asp Asp Met Pro
    130                 135                 140

Met Leu Asn Lys Ile Ala Gln Phe Met Asp Asp Asn Pro Ala Gln Phe
145                 150                 155                 160

Pro Lys Pro Asp Ser Gly Ser Trp Val Asn Glu Leu Lys Glu Asp Asn
                165                 170                 175

Phe Leu Asp Gly Asp Glu Thr Ala Ala Phe Arg Ser Ala Leu Asp Ile
            180                 185                 190

Ile Gly Gln Gln Leu Gly Asn Gln Gln Ser Gly Ala Gly Gly Leu Ala
        195                 200                 205

Gly Thr Gly Gly Gly Leu Gly Thr Pro Ser Ser Phe Ser Asn Asn Ser
    210                 215                 220

Ser Val Thr Gly Asp Pro Leu Ile Asp Ala Asn Thr Gly Pro Gly Asp
225                 230                 235                 240
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Ser Gly Asn Ser Ser Gly Glu Ala Gly Gln Leu Ile Gly Glu Leu Ile
                245                 250                 255

Asp Arg Gly Leu Gln Ser Val Leu Ala Gly Gly Gly Leu Gly Thr Pro
            260                 265                 270

Val Asn Thr Pro Gln Thr Gly Thr Ala Ala Asn Gly Gly Gln Ser Ala
        275                 280                 285

Gln Asp Leu Asp Gln Leu Leu Gly Gly Leu Leu Leu Lys Gly Leu Glu
    290                 295                 300

Ala Thr Leu Lys Asp Ala Gly Gln Thr Ala Thr Asp Val Gln Ser Ser
305                 310                 315                 320

Ala Ala Gln Ile Ala Thr Leu Leu Val Ser Thr Leu Leu Gln Gly Thr
                325                 330                 335

Arg Asn Gln Ala Ala Ala
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<210> SEQ ID NO 4
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<210> SEQ ID NO 6
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<223> OTHER INFORMATION: Synthetic primer derived from the reported PSPAL1 promoter

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<210> SEQ ID NO 7

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<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer derived from the reported PSPAL1 promoter

<400> SEQUENCE: 7

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1. A transgenic, disease-resistant plant which has been transformed with an expression cassette comprising:

a promoter capable of promoting a constitutive, inducible, or organ- or phase-specific gene expression; and

a gene, under the control of said promoter, encoding an elicitor protein;

wherein said plant is capable of effecting the constitutive, inducible, or organ- or phase-specific expression of the elicitor protein in an amount effective for inducing a defense reaction.

2. A transgenic, disease-resistant plant as claimed in claim 1, wherein said promoter capable of promoting a constitutive, inducible, or organ- or phase-specific gene expression and said gene, under the control of said promoter, encoding an elicitor protein, are integrated into the genome.

3. A transgenic, disease-resistant plant as claimed in claim 1 or **2**, wherein said elicitor protein is a protein possessing a hypersensitive-response-inducing activity against disease microorganisms.

4. A transgenic, disease-resistant plant as claimed in claim 3, wherein said protein possessing a hypersensitive-response-inducing activity is selected from:

(a) a protein consisting of the amino acid sequence of SEQ. ID No. 2;

(b) a protein consisting of an amino acid sequence derived from the amino acid sequence of SEQ. ID No. 2 by deletion, substitution, addition or insertion of one or more amino acids, and possessing a hypersensitive-response-inducing activity; and

(c) a protein consisting of an amino acid sequence being at least 50% homologous to the amino acid sequence of SEQ. ID No. 2, and possessing a hypersensitive-response-inducing activity.

5. A transgenic, disease-resistant plant as claimed in claim 2, wherein said gene encoding an elicitor protein is selected from:

(a) a DNA molecule consisting of the nucleotide sequence of SEQ. ID No. 1;

(b) a DNA molecule consisting of a nucleotide sequence derived from the nucleotide sequence of SEQ. ID No. 1 by deletion, substitution, addition or insertion of one or more nucleotides, and encoding a protein possessing a hypersensitive-response-inducing activity;

(c) a DNA molecule consisting of a nucleotide sequence being hybridizable with a DNA molecule consisting of the complementary nucleotide sequence to the nucleotide sequence of SEQ. ID No. 1 under stringent conditions, and encoding a protein possessing a hypersensitive-response-inducing activity; and

(d) a DNA molecule consisting of a nucleotide sequence being at least 50% homologous to the nucleotide sequence of SEQ. ID No. 1, and encoding a protein possessing a hypersensitive-response-inducing activity.

6. A method for producing a transgenic, disease-resistant plant capable of effecting a constitutive, inducible, or organ- or phase-specific expression of an elicitor protein in an amount effective for inducing a defense reaction, comprising the steps of:

(a) obtaining a transgenic plant cell with an expression cassette comprising a promoter capable of promoting a constitutive, inducible, or organ- or phase-specific gene expression and a gene, under the control of said promoter, encoding an elicitor protein; and

(b) reconstructing, from said transgenic plant cell, a complete plant.

7. An expression cassette for producing a transgenic, disease-resistant plant capable of effecting a constitutive, inducible, or organ- or phase-specific expression of an elicitor protein in an amount effective for inducing a defense reaction, comprising at least:

(a) a promoter capable of promoting a constitutive, inducible, or organ- or phase-specific gene expression; and

(b) a gene, under the control of said promoter, encoding the elicitor protein.

8. An expression cassette as claimed in claim 7, wherein said elicitor protein is a protein possessing a hypersensitive-response-inducing activity against disease microorganisms.

9. An expression cassette as claimed in claim 8, wherein said protein possessing a hypersensitive-response-inducing activity is selected from:

(a) a protein consisting of the amino acid sequence of SEQ. ID No. 2;

(b) a protein consisting of an amino acid sequence derived from the amino acid sequence of SEQ. ID No. 2 by deletion, substitution, addition or insertion of one or more amino acids, and possessing a hypersensitive-response-inducing activity; and

(c) a protein consisting of an amino acid sequence being at least 50% homologous to the amino acid sequence of SEQ. ID No. 2, and possessing a hypersensitive-response-inducing activity.

10. An expression cassette as claimed in claim 7, wherein said gene encoding an elicitor protein is selected from:

(a) a DNA molecule consisting of the nucleotide sequence of SEQ. ID No. 1;

(b) a DNA molecule consisting of a nucleotide sequence derived from the nucleotide sequence of SEQ. ID No. 1 by deletion, substitution, addition or insertion of one or more nucleotides, and encoding a protein possessing a hypersensitive-response-inducing activity;

(c) a DNA molecule consisting of a nucleotide sequence being hybridizable with a DNA molecule consisting of the complementary nucleotide sequence to the nucleotide sequence of SEQ. ID No. 1 under stringent conditions, and encoding a protein possessing a hypersensitive-response-inducing activity; and

(d) a DNA molecule consisting of a nucleotide sequence being at least 50% homologous to the nucleotide sequence of SEQ. ID No. 1, and encoding a protein possessing a hypersensitive-response-inducing activity.

11. An expression cassette as claimed in any one of claims **7-10** for producing a transgenic, systemic acquired disease-resistant plant.

12. An expression cassette as claimed in any one of claims **7-11**, wherein said elicitor protein is expressed specifically at the time of infection of disease microorganisms in an amount effective for inducing a defense reaction.

13. An expression cassette as claimed in claim 12, comprising a constitutive, or organ- or phase-specific promoter.

14. A recombinant vector carrying an expression cassette as claimed in any one of claims **7-13**.

15. A gene consisting of a DNA molecule selected from:

(a) a DNA molecule consisting of the nucleotide sequence of SEQ. ID No. 1;

(b) a DNA molecule consisting of a nucleotide sequence derived from the nucleotide sequence of SEQ. ID No. 1 by deletion, substitution, addition or insertion of one or more nucleotides, and encoding a protein possessing a hypersensitive-response-inducing activity;

(c) a DNA molecule consisting of a nucleotide sequence being hybridizable with a DNA molecule consisting of the complementary nucleotide sequence to the nucleotide sequence of SEQ. ID No. 1 under stringent conditions, and encoding a protein possessing a hypersensitive-response-inducing activity; and

(d) a DNA molecule consisting of a nucleotide sequence being at least 50% homologous to the nucleotide sequence of SEQ. ID No. 1, and encoding a protein possessing a hypersensitive-response-inducing activity.

16. A gene encoding a protein selected from:

(a) a protein consisting of the amino acid sequence of SEQ. ID No. 2;

(b) a protein consisting of an amino acid sequence derived from the amino acid sequence of SEQ. ID No. 2 by deletion, substitution, addition or insertion of one or more amino acids, and possessing a hypersensitive-response-inducing activity; and

(c) a protein consisting of an amino acid sequence being at least 97% homologous to the amino acid sequence of SEQ. ID No. 2, and possessing a hypersensitive-response-inducing activity.

17. A protein selected from:

(a) a protein consisting of the amino acid sequence of SEQ. ID No. 2;

(b) a protein consisting of an amino acid sequence derived from the amino acid sequence of SEQ. ID No. 2 by deletion, substitution, addition or insertion of one or more amino acids, and possessing a hypersensitive-response-inducing activity; and

(c) a protein consisting of an amino acid sequence being at least 97% homologous to the amino acid sequence of SEQ. ID No. 2, and possessing a hypersensitive-response-inducing activity.

18. A transgenic, disease-resistant plant as claimed in any one of claims **1-5**, which has been transformed with an expression cassette comprising a constitutive or inducible promoter;

wherein said plant is a transgenic, powdery mildew-resistant tobacco.

19. A transgenic, disease-resistant plant as claimed in any one of claims **1-5**, which has been transformed with an expression cassette comprising a constitutive promoter;

wherein said plant is a transgenic, blast-resistant rice.

* * * * *

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A plant signal sequence enhances the secretion of bacterial ChiA in transgenic tobacco

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Key words: chitinase, glycosylation, protein secretion, transgenic plants

Abstract

When the secreted bacterial protein ChiA is expressed in transgenic tobacco, a fraction of the protein is glycosylated and secreted from the plant cells; however most of the protein remains inside the cells. We tested whether the efficiency of secretion could be improved by replacing the bacterial signal sequence with a plant signal sequence. We found the signal sequence and the first two amino acids of the PR1b protein attached to the ChiA mature protein directs complete glycosylation and secretion of the ChiA from plant cells. Glycosylation of this protein is not required for its efficient secretion from plant cells.

Introduction

In eukaryotes most secreted proteins have been shown to possess a signal sequence of approximately thirty amino acids at the N-terminus, which when recognized by the appropriate cellular machinery leads to the translocation of the protein across the membrane of the endoplasmic reticulum [15]. Signal sequences show little homology at the amino acid level but do share common features including positive charge at the amino-terminus, an internal stretch of hydrophobic amino acids, and a polar carboxy-terminal region which contains the cleavage site [22]. These features are conserved in the eukaryotic and prokaryotic kingdoms with some signal sequences across kingdom boundaries [20, 24].

We have shown previously that when the *Serratia marcescens chiA* gene (which codes for a secreted protein, ChiA) is expressed in tobacco cells, a fraction of the expressed protein is modified by the attachment of complex glycans and secreted from plant cells [12]. In this paper, we describe experiments directed towards improving secretion of ChiA by plant cells. We have tested whether secretion depends upon the presence of a N-terminal signal sequence and if replacement of the signal sequence of ChiA with that of the tobacco PR1b protein increases secretion of the ChiA protein by plant cells. The secretion of mutated forms of ChiA lacking the consensus sequence for N-linked glycosylation was also investigated.

Materials and methods

Plasmid construction

The pChiA plant transformation series derivatives were all prepared in the binary plasmid pJJ2964. This plasmid contains T-DNA carrying an *nptII* gene driven by the *nos* promoter (to enable selection of transformed tissue on kanamycin), and unique *Bam* HI and *Hin*d III cloning sites. Manipulations on the *chiA* gene were carried out with it cloned in the vector pUC118 as a fragment containing the cauliflower mosaic virus (CaMV) 35S promoter followed by a leader from the petunia *Cab22L* gene [6], upstream from the complete *chiA* gene from *Serratia marcescens*. Downstream from the *chiA* gene is a fragment carrying the polyadenylation signals from the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene. The *chiA* gene had the following modifications to its sequence [7]: (1) a novel *Nco* I site at position + 1; (2) a novel *Sma* I site at position 78; (3) the *Sma* I site present in the native sequence at position 951 has been removed. Oligonucleotide-directed mutagenesis was used to make all these changes. The novel *Nco* I site changes the second amino acid in the signal peptide from Arg to Ala; the other changes have no effect on the protein sequence.

To construct the plasmid pChiA, the *Bgl* II-*Hin*d III fragment from the pUC118 derivative described above was ligated into *Bam* HI-*Hin*d III-cut pJJ2964. The plasmid pChiA-M was constructed following oligonucleotide loop-out mutagenesis of the *chiA* gene cloned in pUC118, which removed all the codons of the ChiA signal sequence (amino acids 2 to 23) except for the initiator methionine. The *Bgl* II-*Hin*d III fragment carrying the modified *chiA* gene and the plant expression signals was then ligated into pJJ2964 as described for pChiA to form pChiA-M.

The plasmid pPRSSChiA was constructed by synthesizing the codons for the PR1b signal sequence plus the first two amino acids of the mature PR1b protein as two complementary oligonucleotides, with a half *Nco* I site at the 5′ end and a half *Sma* I site at the 3′ end. This was ligated into the *Nco* I and *Sma* I sites at the 5′ end of the *chiA* gene. pPRSSChiA was then constructed by ligating the *Bgl* II-*Hin*d III fragment into pJJ2964, as described above for the other pChiA plasmids.

To remove the glycosylation sites from the ChiA protein, the codons for amino acids at potential N-glycosylation sites (Asn-X-Ser/Thr) were identified on the DNA sequence, then oligonucleotide-directed mutagenesis was used to change the codon for the third amino acid in each site to alanine. All manipulations were carried out on the *chiA* gene cloned in pUC118 and all changes were verified by DNA sequence analysis. The plasmids pChiA-G and pPRSSChiA-G were then constructed; these are identical to pChiA and pPRSSChiA except that both contain all four of the site-directed mutations that remove the four consensus glycosylation sites.

Plant cell tissue culture

Plant transformations, establishment, maintenance and sampling of suspension cultures, and protoplast preparations were as described [12]. All plant transformations were carried out using *Nicotiana tabacum* cv. SR1.

Protein extraction and measurement

Protein extractions, electrophoresis and immunoblotting of protein extracts were all carried out as described [12], except that immunoblots were developed using an alkaline phosphatase conjugate in place of the horseradish peroxidase conjugate. The buffer used for making protein extracts for gel and enzyme analysis contained 84 mM sodium citrate, 32 mM sodium phosphate, 6 mM ascorbic acid, and 14 mM β-mercaptoethanol, pH 5.5

Nucleic acid analysis

DNA manipulations were carried out as described [13] or according to enzyme supplier

instructions. RNA extraction from leaf tissue and primer extension analysis for the quantification of steady-state RNA and confirmation of transcription start sites was carried out as described [6]. Oligonucleotide-directed mutagenesis was by the method of Kunkel [11]; all changes were confirmed by DNA sequence analysis as described by Sanger *et al.* [17]. Oligonucleotide primers for mutagenesis and sequence analysis were made on an Applied Biosystems 381A DNA synthesizer.

Results

To test whether the bacterial signal sequence of ChiA is required for plant cell secretion, we prepared a deletion mutant of the *chiA* gene lacking the region which specifies the codons of the signal sequence, pChiA-M (amino acids 2 to 23); the amino terminal of the resulting protein from pChiA-M is shown in Fig. 1. The ChiA protein was then expressed in plant cells with and without its signal sequence by transformation with the binary plasmids pChiA and pChiA-M. In parallel, to determine whether the fraction of ChiA secreted by tobacco cells could be increased by fusion to the signal sequence from a secreted plant protein, we constructed a translational fusion between PR1b and the mature ChiA protein. We chose the tobacco PR1b protein as the source of a plant signal sequence because complete sequence information was available for the PR1b gene and the extracellular location of the protein has been well studied. The portion of the *chiA* gene encoding the signal sequence of ChiA was replaced with that encoding the signal sequence from PR1b so that the resulting fusion protein contains the PR1b signal sequence plus the first two amino acids of the PR1b mature protein (Gln-Asn) in place of the first two amino acids of the mature ChiA protein (Ala-Ala) (see Fig. 1). This fusion protein was also expressed in plant cells by transformation with the binary plasmid pPRSSChiA. At least 10 independent transformants were prepared for each of the constructions pChiA, pChiA-M and pPRSSChiA, then 2 plants from each group were selected for subsequent comparative analyses. The representative plants were chosen so that the transformants carrying the different *chiA* genes each were expressing similar steady-state *chiA* mRNA levels and ChiA protein.

Immunoblots of leaf proteins isolated from two plants transformed with pChiA show multiple bands (Fig. 2, lanes 2 and 3), the most prominent of which comigrates with ChiA protein expressed in *Escherichia coli* (Fig. 2, lane 1). We have previously shown that the most prominent species is an intracellular form, and the fainter bands of higher molecular weight are glycosylated, secreted forms of ChiA [12]. Immunoblots of protein from two plants transformed with pChiA-M (Fig. 2, lanes 6 and 7) show a single band which comigrates with ChiA from *E. coli*; no species of higher molecular weight can be detected, which indicates that glycosylation of ChiA does not occur when it is expressed without a signal sequence. Immunoblot analysis of leaf extracts from plants transformed with pPRSSChiA shows that, in contrast to those transformed with pChiA, all of the cross-

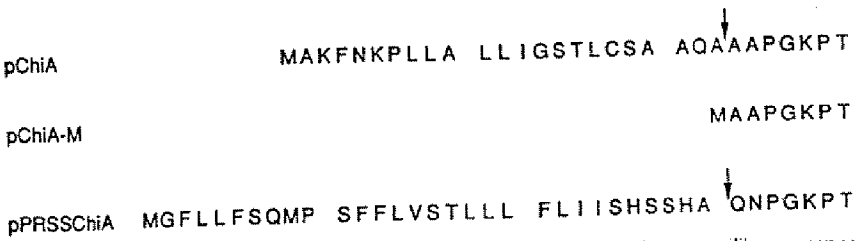

Fig. 1. Deduced amino acid sequences of the deletion and fusion derivatives of the *chiA* gene. The sequences in each case extend to the 7th amino acid in the mature ChiA sequence. The vertical arrow indicates the likely site of cleavage of the signal peptide (known for ChiA in *E. coli* and predicted for PRSS on the basis of the known cleavage point in the PR1b protein).

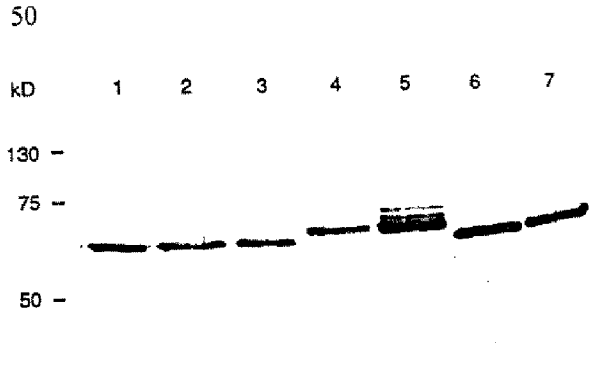

Fig. 2. Immunoblot with ChiA antibody to the total leaf protein (100 μg) from individual tobacco plants transformed with ChiA derivatives. Lane 1: ChiA from *E. coli* (200 ng); lanes 2 and 3: pChiA (ChiA signal sequence); lanes 4 of 5: pPRSS-ChiA (PR1 signal sequence); lanes 6 and 7; pChiA-M (no signal sequence).

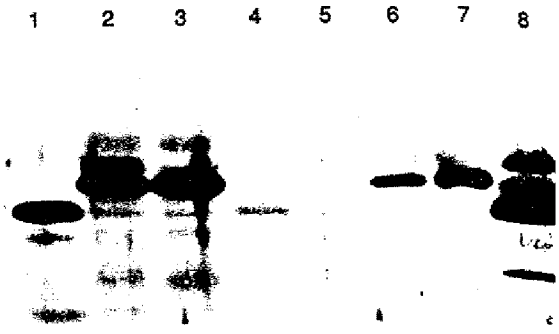

Fig. 3. Immunoblot with ChiA antibody to protein isolated from suspension culture medium. Lane 1: ChiA protein from *E. coli* (200 ng); lanes 2 and 3: medium from pPRSSChiA cells; lanes 4 and 5: medium from pChiA-M cells; lanes 6 and 7: medium from pChiA cells (lanes 2–7 each contain protein extracted from 1 ml culture medium); lane 8: 100 μg leaf protein from ChiA plant.

reacting protein is in a position corresponding to the glycosylated forms of ChiA (Fig. 2, lanes 4 and 5).

We assayed the level of secretion of ChiA from these different transgenic plant cells by analyzing the media from plant cell suspension cultures and by comparing the profiles of protein extracts from leaf protoplasts and corresponding whole leaves. We have shown that these approaches give consistent results and correctly demonstrate secretion of the PR1b secreted tobacco protein (unpublished data). The culture fluid from suspension cultures established from individual plants transformed with pChiA, pChiA-M or pPRSSChiaA, was analyzed by immunoblotting (Fig. 3). There is little or no ChiA protein in the medium from the pChiA-M transformed cells (lanes 4 and 5), and high levels of ChiA in the medium from the pPRSSChiA or pChiA transformed cells (lanes 2, 3, 6 and 7). Furthermore the ChiA which is present in the culture medium from pPRSSChiA and pChiA transformants is the higher-molecular-weight glycosylated form. The faint band seen in lanes 4 and 5, which comigrates with the bacterial standard (lane 1), probably corresponds to non-glycosylated non-secreted ChiA which is in the culture fluid as a consequence of cell death. These data from the analysis of suspension culture media suggest that in the absence of any signal sequence (pChiA-M) the ChiA which is expressed is not secreted. In the presence of signal sequence, either the ChiA signal (pChiA or the PR1b signal (pPRSSChiA), ChiA protei is glycosylated and secreted. The observation th higher levels of ChiA protein appear in the m dium from pPRSSChiA transformants (Fig. lanes 2, 3) than from pChiA transforman (Fig. 3, lanes 6, 7) suggests that secretion is mo efficient when the PR1b signal is fused to Chi

Since secreted proteins will be present in le tissues extracts but absent from washed prot plasts, we have compared these tissues from t transgenic plants to further determine whetl secretion is occurring. The results from typi experiments comparing these two tissues shown in Fig. 4. In the total leaf extract fron plant transformed with pCHiA (lane 7), differ molecular weight forms of the ChiA protein be seen which correspond to glycosylated (upp and non-glycosylated (lower) forms of the p tein. In washed protoplasts of these plants, c the non-glycosylated (lower) form of ChiA is s (lane 6), which is consistent with our above re indicating that the glycosylated higher-molecu weight forms are secreted from plant cells plants transformed with pChiA-M, the prc profiles of washed protoplasts (lane 2) and t leaf extract (lane 3) are identical and corresp to the *E. coli* standard (lane 1), indicating

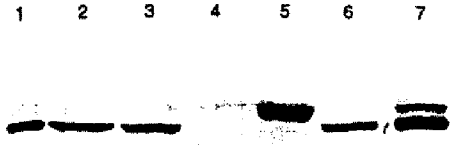

Fig. 4. Immunoblot of protein from leaf or from washed protoplasts. Lane 1: ChiA from *E. coli* (200 ng); lanes 2 and 3: pChiA-M; lanes 4 and 5: pPRSSChiA; lanes 6 and 7: pChiA. Lanes 2, 4 and 6 are from protoplasts; lanes 3, 5 and 7 are from leaf. Lanes 2 to 7 each contain 100 μg total protein.

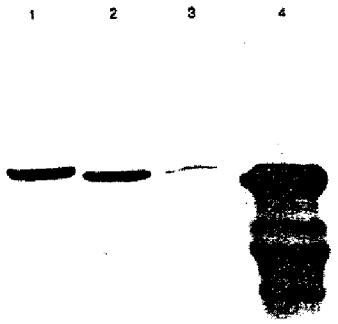

Fig. 5. Immunoblot of protein from protoplast and leaf extracts from plants expressing ChiA lacking glycosylation sites. Lane 1: *E. coli* ChiA (150 ng); lanes 2 and 3: pChiA-G; lanes 4 and 5: pPRSSChiA-G. Lanes 2 to 5 each contain 50 μg total protein; lanes 2 and 4 are leaf extracts, lanes 3 and 5 are protoplast extracts.

glycosylation has not taken place and that little or no secretion is occurring. The profiles from washed protoplasts and total leaf extracts of pPRSSChiA transformants are shown in lanes 4 and 5, and in this case the ChiA protein is present solely as a higher-molecular-weight form, none of which is detected inside washed protoplasts. These data comparing proteins from protoplasts and total leaf extracts confirm that secreted and glycosylated forms of ChiA protein occur only if a signal sequence is attached, and if the PR1b signal sequence is used then all of the ChiA protein is secreted and glycosylated.

There are four consensus N-glycosylation sites (Asn-X-Ser/Thr) in the predicted ChiA protein sequence. We constructed a derivative of the *chiA* gene where all four sites were 'inactivated' by altering the last codon in the consensus site to Ala. To express the mutant ChiA proteins in plant cells, the binary plasmids pChiA-G and pPRSSChiA-G were used to produce transformed tobacco plants. RNA and protein analysis was used to identify plants expressing high levels of the mutant ChiA proteins. (We noted that expression at both the RNA and protein level was generally significantly higher for plants transformed with pPRSSChiA-G than pChiA-G.)

We compared the protein profile in washed protoplasts with that in total leaf extracts from pChiA-G and pPRSSChiA-G transformants (Fig. 5). The ChiA protein in all plant extracts co-migrated with the mature ChiA protein as purified from bacteria, as would be predicted if no glycosylation were occurring. In pChiA-G transformants, the intensity of the ChiA band was greater in total leaf extract (lane 2) than in protoplast extracts (lane 3), suggesting that, as with the wild-type protein, secretion of ChiA does occur but not all of the ChiA is being secreted from the cells. In pPRSSChiA-G transformants, a trace of ChiA was detected in the protoplast extracts (lane 5) in contrast to the very high levels in total extract from leaf (lane 4), indicating that most or all of the unglycosylated ChiA protein is being secreted.

Discussion

We have investigated the secretion of the bacterial ChiA protein from plant cells. We had previously demonstrated that ChiA fused to the bacterial signal sequence is inefficiently secreted by plant cells. Here we show that the ChiA protein is fully secreted when the signal sequence derived from the tobacco PR1b protein is fused to the ChiA mature protein and secretion does not occur in the absence of a signal sequence. The lack of secretion in the absence of a signal sequence is expected, given the role of signal sequences in mediating targeting of proteins to the lumen of the endoplasmic reticulum in eukaryotic cells [16].

The fact that no detectable glycosylation of ChiA occurs in the absence of a signal sequence is also expected, since the initial transfer of glycans to proteins occurs as the proteins cross the ER membrane [9].

The most likely explanation for the improved efficiency of ChiA secretion in pPRSSChiA transformants is that the possession of a plant signal sequence improves the ability of the ChiA protein to enter the secretory pathway of the plant cells in which it is expressed. An alternative explanation is that the mature ChiA peptides arising from pChiA- and pPRSSChiA-transformed plants differ in the two N-terminal amino acids, and this difference could alter the mature protein so that it would behave differently in the plant secretory pathway. While this explanation cannot be ruled out, we believe it to be less likely, as we have not detected any significant differences in the physical or enzymological properties of the ChiA expressed with a bacterial or a plant signal sequence. (The precise point of cleavage of the signal sequences when expressed in plant cells remains to be determined.)

Signal sequences show considerable degeneracy, so that even random peptide sequences can function as signal sequences [8]; however, significant differences between prokaryotic and eukaryotic signal sequences are revealed when large numbers of sequences are analyzed statistically [23]. These differences may be reflected in the ability of signal sequences to function efficiently in heterologous hosts. There are reports where the use of a signal sequence native to the organism in which the protein is being expressed can enhance the secretion of a heterologous protein [1, 2, 19], and others where more efficient secretion of a foreign protein is seen when it possesses its own signal sequence rather than one derived from the organism in which it is expressed [2, 19]. Determining which features of the PR1b N-terminus are relevant in mediating the efficient secretion of ChiA from plant cells would be an interesting area for further study.

The fact that ChiA is apparently completely located outside the cell when expressed with a plant signal sequence may be taken as further evidence that the pathway for secretion in plant cells is a default pathway, requiring no positive sorting information other than the possession of a functional signal sequence. Thus it seems likely that many other proteins could also be engineered to be plant secretory proteins. In support of this, Denecke *et al.* [3] have recently shown that three normally cytoplasmic proteins can be secreted from plant cells by the attachment of a suitable signal sequence.

Glycan side-chains attached to proteins probably have multiple roles [14]; it has often been observed that prevention of glycosylation also prevents the secretion of the altered protein. Sometimes this can be attributed to decreased stability of the altered protein to proteases [4, 14], or to aggregation of the protein [5]. There are also cases where the non-glycosylated forms of the protein are secreted as efficiently as are the glycosylated forms [10, 18]. Thus the role of glycans in intracellular targeting is not simple and cannot be generalized. The likelihood of a direct role for glycan residues in some aspect of protein targeting in eukaryotic cells (for example, by interacting with a receptor as opposed to simply changing the physical properties of the protein) seems remote. Only in the case of lysosome is targeting mediated by mannose-6-phosphate residues [21]. The results presented in this paper demonstrate clearly that the efficiency with which ChiA can be secreted by plant cells is not influenced by the extent to which it is glycosylated.

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The HrpZ Proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* Are Encoded by an Operon Containing *Yersinia ysc* Homologs and Elicit the Hypersensitive Response in Tomato but not Soybean

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The *Pseudomonas syringae* pathovars are composed of host-specific plant pathogens that characteristically elicit the defense-associated hypersensitive response (HR) in nonhost plants. *P. s.* pv. *syringae* 61 secretes an HR elicitor, harpin$_{Pss}$ (HrpZ$_{Pss}$), in a *hrp*-dependent manner. An internal fragment of the *P. s.* pv. *syringae* 61 *hrpZ* gene was used to clone the *hrpZ* locus from *P. s.* pv. *glycinea* race 4 (bacterial blight of soybean) and *P. s.* pv. *tomato* DC3000 (bacterial speck of tomato). DNA sequence analysis revealed that *hrpZ* is the second ORF in a polycistronic operon. The amino acid sequence identities of HrpZ$_{Pss}$/HrpZ$_{Psg}$ and HrpZ$_{Pss}$/HrpZ$_{Pst}$ were 79 and 63%, respectively. Although none of the HrpZ proteins showed significant overall sequence similarity with other known proteins, HrpZ$_{Pst}$ contained a 24-amino acid sequence that is homologous with a region of the PopA1 elicitor protein of the tomato pathogen, *Pseudomonas solanacearum* GMI1000. *hrpA*, the upstream ORF, was highly divergent: The amino acid sequence identities of HrpA$_{Pss}$/HrpA$_{Psg}$ and HrpA$_{Pss}$/HrpA$_{Pst}$ were 91 and 28%, respectively, and no HrpA sequence showed similarity to known proteins. In contrast, the predicted products of the downstream ORFs in *P. s.* pv. *syringae* and *P. s.* pv. *tomato*, *hrpB*, *hrpC*, *hrpD*, and *hrpE* showed varying levels of similarity to those of *yscI*, *yscJ*, *yscK*, and *yscL*. These are colinearly arranged genes in the *virC* locus of *Yersinia* spp., which are involved in the secretion of the Yop virulence proteins via the type III pathway. The similarity of the Ysc proteins was generally stronger in comparisons with the *P. s.* pv. *tomato* Hrp proteins. The HrpZ proteins were purified by heat denaturation of contaminating proteins followed by ammonium sulfate fractionation, hydrophobic chromatography, and gel electrophoresis. All three HrpZ proteins elicited the HR in tomato, whereas none of them elicited significant necrosis in soybean. The results indicate that HrpZ is encoded in an operon containing some of the genes involved in its own secretion and suggest that HrpZ structure does not directly determine bacterial host range.

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Phytopathogenic strains of *Pseudomonas syringae* cause two patterns of necrosis when the bacteria invade a plant. On a susceptible ("compatible") host, a necrotic lesion often develops over a period of days, with necrosis spreading as the bacteria multiply and the plant becomes diseased. On a resistant or nonhost plant, a localized cellular necrosis is induced within 24 to 48 h, and bacterial multiplication is inhibited. This was first reported by Klement (1963; Klement et al. 1964), who observed that when high concentrations of pathogenic bacteria are infiltrated into an incompatible plant they elicit a visible necrosis which is limited to the infiltrated area. This reaction, called the hypersensitive response (HR), involves localized cell death and production of anti-microbial compounds at the site of pathogen invasion (Bonas 1994).The ability of *P. syringae* and other nontumorigenic, gram-negative, bacterial pathogens to elicit the HR is governed by *hrp* genes. Typical Hrp$^-$ mutants are pleiotropically defective in planta: They do not elicit the HR in nonhosts and they fail to multiply and cause disease in host plants (Lindgren et al. 1986). Clusters of *hrp* genes have been identified in many gram-negative phytopathogenic bacteria (Bonas 1994). A 25-kb *hrp* cluster from *P. s.* pv. *syringae* 61 is sufficient to confer the tobacco HR phenotype, but not the pathogenic phenotype on nonpathogenic bacteria (Huang et al. 1988). *hrp* genes have also been cloned and characterized extensively from *P. s.* pv. *phaseolicola* NPS3121, *P. solanacearum* GM1000, *Xanthomonas campestris* pv. *vesicatoria* 75-3, and *Erwinia amylovora* Ea321 (Lindgren et al. 1986; Boucher et al. 1987; Beer et al. 1991; Bonas et al. 1991). Certain *hrp* genes are widely conserved among these pathogens, and several encode components of a protein secretion pathway that is similar to the type III pathway used by *Yersinia*, *Shigella*, and *Salmonella* spp. to secrete extracellular proteins involved in animal pathogenesis (Van Gijsegem et al. 1993). One activity of the *hrp*-encoded secretion pathway in phytopathogenic bacteria is the secretion of proteinaceous elicitors of the HR, which are also encoded by *hrp* genes.

The first *hrp*-encoded elicitor characterized was harpin$_{Ea}$ from *E. amylovora* (Wei et al. 1992). Similar elicitors have since been isolated from other bacteria, including *P. s.* pv. *syringae* 61, *P. solanacearum* GMI1000, and *E. chrysanthemi*

EC16 (He et al. 1993; Arlat et al. 1994; Bauer et al. 1994). Proteins in this family of elicitors share several general characteristics. They are glycine rich, heat-stable, lack cysteine, and appear highly susceptible to proteolysis. They lack an N-terminal signal peptide, but they are secreted to the bacterial milieu. Their expression and secretion is dependent on *hrp* genes. The biological role of these proteins in pathogenesis has not yet been determined, but the purified proteins can induce an HR on a nonhost plant such as tobacco. However, there are significant differences in the organization of the elicitor operons and the activity of the elicitors, which suggests that the *Erwinia* harpins, the *P. syringae hrpZ* product and the *P. solanacearum popA* product may represent three distinct classes of elicitors. In this work we will refer to the *P. s.* pv. *syringae* elicitor as $HrpZ_{Pss}$ rather than $harpin_{Pss}$ (He et al. 1993). This distinction is supported by the weak similarity of the amino acid sequences of the four proteins, with the only exception being the C-terminal halves of the *Erwinia* harpins (Bauer et al. 1994).

The location of known elicitor genes in reference to the *hrp* cluster varies in *P. s.* pv. *syringae*, *P. solanacearum*, and *E. amylovora*. *hrpN* and *hrpZ* are contiguous or within the *hrp* cluster, whereas *popA* lies outside (although near) the *P. solanacearum hrp* cluster (Wei et al. 1992; He et al. 1993; Arlat et al. 1994). There are no genes downstream of the elicitor gene in either the *hrpN* or the *popA* operons, which means that mutations in the elicitor genes do not have a polar effect on the Hrp phenotype, and mutant construction is straightforward. In contrast, mutagenesis and complementation studies of the *hrp* cluster from *P. s.* pv. *syringae* 61 have indicated that *hrpZ* lies upstream of at least one other *hrp* gene within an operon (Huang et al. 1991; Xiao et al. 1992).

In *E. amylovora* and *E. chrysanthemi*, harpins have been demonstrated to be sufficient and necessary to elicit the HR, and mutation of *hrpN* in *E. amylovora* has shown that $harpin_{Ea}$ is required for pathogenesis (Wei et al. 1992). However *hrpN* mutants of *E. chrysanthemi* can establish infections, albeit at a significantly reduced frequency, which suggests that $harpin_{Ech}$ is important but not essential for pathogenesis (Bauer et al. 1995). In contrast, a *popA* mutant of *P. solanacearum* is fully pathogenic on susceptible hosts, indicating that PopA1 is not required for pathogenesis (Arlat et al. 1994).

These elicitors may play a role in controlling the host specificity exhibited by *E. amylovora* and plant pathogenic pseudomonads such as *P. syringae* and *P. solanacearum*. However it is difficult to compare the activity of $HrpZ_{Pss}$ and $harpin_{Ea}$ in host and nonhost plants because legumes and rosaceous plants, the hosts of *P. s.* pv. *syringae* 61 and *E. amylovora* Ea321, respectively, respond poorly to preparations of any of these elicitor proteins (Wei et al. 1992; He et al. 1993). PopA1 from *P. solanacearum* does appear to act in a host-specific manner, inducing an HR on resistant lines of petunia and the nonhost tobacco, but not on susceptible lines of petunia or tomato (Arlat et al. 1994). This phenotype is similar to that of *avr* genes, but PopA1 is distinct from known Avr proteins in eliciting the HR directly on resistant plants. $Harpin_{Ech}$ elicits an HR on some compatible hosts of *E. chrysanthemi*, but in contrast to the other three bacteria *E. chrysanthemi* is a broad-host range pathogen and the activity of $harpin_{Ech}$ may not be representative of elicitor activity in a highly host-specific system (Bauer et al. 1995).

In previous work we cloned and characterized the *hrpZ* gene from *P. s.* pv. *syringae* 61, a weak pathogen of bean, and demonstrated with Southern and immunoblots that other pathovars of *P. syringae* contain homologs of this gene (He et al. 1993). This supported the hypothesis that HrpZ represents a family of elicitors common to all pathogenic strains of *P. syringae*. We report here the isolation of homologs of $HrpZ_{Pss}$ from two other experimentally important pathovars of *P. syringae-P. s.* pv. *tomato* and *P. s.* pv. *glycinea*. Examining HrpZ from these three pathovars enabled us to look within this family of elicitors for variations in sequence and activity which could indicate a role in host range determination. In addition, we characterized the two genes flanking *hrpZ* in *P. s.* pv. *syringae* and *P. s.* pv. *glycinea* and the entire *hrpZ* operon of *P. s.* pv. *tomato*. In conjunction with an accompanying paper (Huang et al. 1995), this completes the sequence of the *P. s.* pv. *syringae* 61 *hrp* genes carried on pHIR11 and provides clues to the function of the genes downstream of *hrpZ*. A preliminary account of portions of this work has been published (Collmer et al. 1994).

RESULTS

Cloning *hrpZ* from *P. s.* pv. *tomato* and *P. s.* pv. *glycinea*.

We previously used Southern hybridization to demonstrate that both *P. s.* pv. *glycinea* race 4 and *P. s.* pv *tomato* DC3000 contain sequences homologous to a 0.75 kb *Bst*XI internal fragment of *hrpZ* from *P. s.* pv. *syringae* (He et al. 1993). The same probe was used to screen genomic libraries of *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*. The libraries were constructed in *E. coli* DH5α by inserting 8- to 12-kb fragments from partial *Sau*3AI digests of genomic DNA into the *Bam*HI site of pUCP19. The screen identified two plasmids with inserts of approximately 10 kb: pCPP2201 (*P. s.* pv. *tomato*) and pCPP2200 (*P. s.* pv. *glycinea*). The same *Bst*XI fragment was used to probe a Southern blot of pCPP2201 and pCPP2200 digested with *Bam*HI, *Eco*RI, and *Pst*I. The probe identified two *Pst*I fragments of 2.2 and 2.4 kb from pCPP2201 and pCPP2200 respectively (Fig. 1). The two *Pst*I fragments were cloned into the *Pst*I site of pBluescript II SK(−) (Stratagene, La Jolla, CA) in *E. coli* DH5α to create the plasmids pCPP2202 to pCPP2205, with the inserts in both orientations with respect to the *lac* promoter. Cell lysates of *E. coli* DH5α containing pCPP2203 ($hrpZ_{Pst}$ in the vector promoter orientation) and pCPP2202 ($hrpZ_{Psg}$ in the vector promoter orientation) induced an HR on tobacco, but those from cells containing pCPP2205 ($hrpZ_{Pst}$ in the opposite orientation of the vector promoter) and pCPP2204 ($hrpZ_{Psg}$ in the opposite orientation of the vector promoter) did not. HR activity was retained after incubating the lysate for 10 min at 100°C and removing denatured proteins by centrifugation. Insensitivity to heat treatment is a characteristic feature of previously isolated HR elicitors. Proteins in the lysates were separated on an SDS-polyacrylamide gel, transferred to an Immobilon-P membrane and immunoblotted with antibodies raised against purified $HrpZ_{Pss}$. Cross-reacting proteins of a similar size to $HrpZ_{Pss}$ were observed and provisionally named $HrpZ_{Psg}$ and $HrpZ_{Pst}$ (Fig. 2, lanes 2 and 4).

The intensity of the $HrpZ_{Psg}$ and $HrpZ_{Pst}$ bands was quite low in comparison to the band for $HrpZ_{Pss}$ expressed from pSYH10 in *E. coli* DH5α (Fig. 2, lane 1). This implied either

that expression was low due to the distance of the cloned gene from the *lac* promoter or that $HrpZ_{Psg}$ and $HrpZ_{Pss}$ did not hybridize strongly to the antibodies. A band corresponding to $HrpZ_{Pss}$ from pSYH10 could be clearly seen on a Coomassie-stained gel, but the bands for $HrpZ_{Psg}$ and $HrpZ_{Pss}$ were indistinct, which implies that low expression was a primary reason for the low signal. In an attempt to improve the level of expression of $HrpZ_{Psg}$ and $HrpZ_{Pss}$ we subcloned *Eco*RI-*Bam*HI fragments containing the inserts from pCPP2202 and pCPP2203 behind the T7 promoter of pET21(+) in *E. coli* BL21(DE3) to create the plasmids pCPP2206 and pCPP2207. The T7 promoter enabled a moderate improvement in protein expression (Fig. 2, lanes 3 and 5).

A common arrangement of ORFs in the *hrpZ* operons of *P. s.* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato* revealed by DNA sequence analysis.

Previously, we determined the complete nucleotide sequence of *hrpZ* from *P. s.* pv. *syringae* by sequencing a 1.4-kb subclone of pHIR11 (a cosmid containing the entire *hrp* cluster from *P. s.* pv. *syringae*) (He et al. 1993). In addition, analysis of the complementation groups and transcriptional

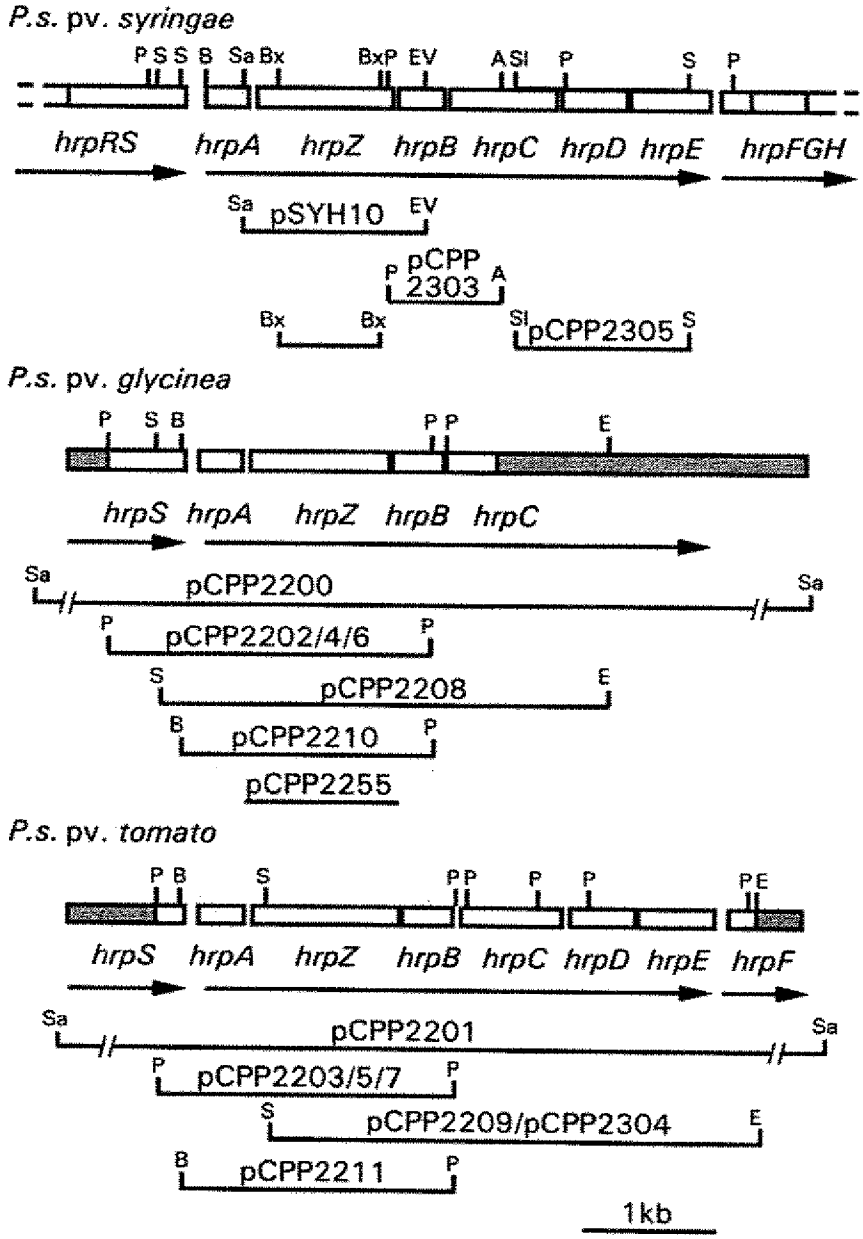

Fig. 1. Physical maps of the *hrpZ* regions from *Pseudomonas syringae* pv. *syringae* 61, *P. s.* pv. *glycinea* race 4, and *P. s.* pv. *tomato* DC3000 and clones used in this study. Open boxes represent sequenced ORFs; filled boxes represent unsequenced DNA. Direction of transcription is indicated by the arrows. Key restriction sites within the sequenced regions are indicated, along with the subclones used in this study. The 0.75-kb *Bst*XI fragment from $hrpZ_{Pss}$ used as a probe for *hrpZ* genes in other pathovars is also shown. Restriction endonuclease abbreviations: A, *Age*I*; B, *Bgl*II; Bx, *Bst*XI*; E, *Eco*RI; EV, *Eco*RV*; P, *Pst*I; S, *Sac*I; Sa, *Sau*3A*; Sl, *Sal*I*. * Not all sites are shown.

units of pHIR11 using Tn*phoA* and Tn5-*gus*A1 mutagenesis (Huang et al. 1991; Xiao et al. 1992) suggested that *hrpZ* lay within an operon, upstream of at least one other *hrp* gene. Further subclones of pHIR11 were used to determine the sequence of the entire $hrpZ_{Pss}$ operon (this study, Huang et al. 1995). We also determined the sequence of (i) the 2.2- and 2.4-kb *Pst*I subclones from pCPP2201 ($hrpZ_{Psi}^{+}$) and pCPP2200 ($hrpZ_{Psg}^{+}$), (ii) an overlapping 3.7-kb *Sac*I-*Eco*RI subclone from pCPP2201 (designated pCPP2209), and (iii) part of an overlapping 3.6-kb subclone from pCPP2200 (designated pCPP2208), as shown in Figure 1. This yielded the sequence of the entire *P. s.* pv. *tomato hrpZ* operon and the first half of the *P. s.* pv. *glycinea* operon. The sequenced region of *P. s.* pv. *syringae* and *P. s.* pv. *tomato* extends from *hrpS* (Xiao et al. 1994), through the *hrpZ* operon to the beginning of the *hrpH* operon (Huang et al. 1992), demonstrating that the organization of this region of the *hrp* cluster is conserved in both pathovars.

Codon preference analysis of the DNA sequence, using *P. s.* pv. *syringae* codon usage data, predicted that *hrpZ* was the second of six ORFs, all oriented in the same direction, an arrangement conserved in *P. s.* pv. *tomato* and at least the first four ORFs of *P. s.* pv. *glycinea*. The sequence of the noncoding DNA is shown in Figure 3. Five of the six ORFs have clear potential ribosome binding sites. The fifth ORF has a putative ribosome binding site in *P. s.* pv. *syringae*, but the site in *P. s.* pv. *tomato* is less clear, the initiation codon shown being selected by alignment with the ORF in *P. s.* pv. *syringae*. In the absence of recognizable terminator elements downstream of the first five ORFs it seems likely that the six ORFs represent a single operon, transcribed from upstream of the first ORF. The five predicted ORFs were provisionally named *hrpA* through *hrpE*, as shown in Figures 1 and 3.

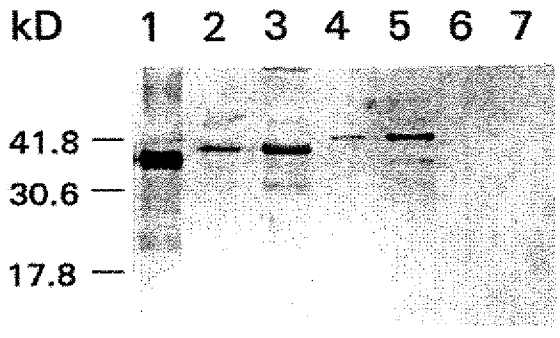

Fig. 2. Immunoblot showing expression of cloned *hrpZ* in *E. coli*. Cultures were grown in LM to an OD_{600} of 0.8 to 1.0 at 30°C, collected by centrifugation and resuspended in 5 mM MES, pH 5.5. For lanes 3, 5 and 7, and 4, T7 expression was induced with 1 mM IPTG when the cells reached an OD_{600} of 0.6, 3 h prior to collection. The cells were disrupted by sonication, and the crude lysate was partially purified by removal of the insoluble fraction after incubating the samples at 100°C for 10 min. SDS-loading buffer was added and the samples were incubated at 100°C for 2 min. The proteins were resolved by SDS-polyacrylamide gel electrophoresis. Following electrophoresis the proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA), probed with anti-$HrpZ_{Pss}$ antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase. Lanes: 1, *E. coli* DH5α (pSYH10) ($HrpZ_{Psy}$); 2, *E. coli* DH5α (pCPP2202)($HrpZ_{Psg}$) 3, *E. coli* BL21(DE3) (pCPP2206)($HrpZ_{Psg}$); 4, *E. coli* DH5α (pCPP2203)($HrpZ_{Psi}$); 5, *E. coli* BL21(DE3) (pCPP2207)($HrpZ_{Psi}$); 6, *E. coli* DH5α (pBluescript II); 7, *E. coli* BL21(DE3)(pET21+).

A *hrp/avr* promoter consensus sequence lies upstream of the *hrpZ* operons of the three *P. syringae* pathovars.

The conserved sequence GGAACC—16bp—CCACNNA lies 50 bp upstream of the initiation codon of *hrpA* in all three pathovars (Fig. 3). This motif has been identified in the promoter regions of many *avr* and *hrp* genes (Innes et al. 1993; Shen and Keen 1993), and appears to be involved in positive regulation by HrpL, a putative alternative sigma factor which is itself positively regulated by HrpR and HrpS (Xiao and Hutcheson 1994). HrpL is a member of a family of alternative sigma factors, many of which are involved in secretion of extracellular factors in response to environmental stimuli (Lonetto et al. 1992). The presence of this promoter motif further supports the suggestion that the six ORFs form a single transcriptional unit which is regulated in a *hrp*-dependent manner. This motif can also be found beyond *hrpE*, upstream of *hrpFGH* in *P. s.* pv. *syringae* and *P. s.* pv. *tomato*, as indicated at the bottom of Figure 3, suggesting that the latter three ORFs form an independent *hrp*-regulated transcriptional unit in these two pathovars.

Comparison of the HrpZ proteins of the three *P. syringae* pathovars.

The predicted amino acid sequences for HrpZ from each of the three pathovars are aligned in Figure 4. Although the proteins migrate slightly anomalously on an SDS polyacrylamide gel, the relative sizes of the estimated molecular weights correspond to the predicted values, with $HrpZ_{Psi}$ being the largest of the three proteins (36.5 kDa), followed by $HrpZ_{Psg}$ (35.3 kDa) and $HrpZ_{Pss}$ (34.7 kDa). Amino-terminal sequencing of the first 10 to 15 residues of purified $HrpZ_{Psg}$ and $HrpZ_{Psi}$ confirmed the predicted initiation codons of both proteins, which aligned with the start codon of $HrpZ_{Pss}$ as shown in Figures 3 and 4. The proteins expressed in *E. coli* appear to be the same size as those recovered from the supernatants of *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*, indicating that the cloned gene is intact and that there are no large post-translational modifications or deletions of HrpZ taking place in *P. syringae* but not in *E. coli*.

The amino acid sequence of $HrpZ_{Psg}$ is quite highly conserved with respect to $HrpZ_{Pss}$, having 87% similarity and 79% identity. $HrpZ_{Psi}$ is less conserved with respect to the two other proteins, with 75% similarity and 63% identity to $HrpZ_{Pss}$. However, the physical features of $HrpZ_{Psg}$ and $HrpZ_{Psi}$ are almost identical to those reported for $HrpZ_{Pss}$ (He et al. 1993). All three are glycine-rich proteins lacking cysteine and tyrosine. $HrpZ_{Psi}$ is the most glycine rich, being 15.7% glycine. The proteins lack the hydrophobic signal sequence used to target proteins for secretion via the Sec export pathway (Pugsley 1989). Analysis of the amino acid sequence fails to identify any obviously significant secondary structure, which is consistent with their sensitivity to proteases, and supports the suggestion that they adopt a fairly open structure in aqueous solution.

In our previous analysis of $HrpZ_{Pss}$ (He et al. 1993), we noted the presence of two sets of short, direct repeats. Only one of these repeats, GGGLGTP, is conserved in the three proteins, with the substitution of a serine for threonine in the first repeat of both $HrpZ_{Psg}$ and $HrpZ_{Psi}$. The significance of these repeats, if any, is unknown. A database search with each of the three proteins using the BLAST algorithm (Altschul et

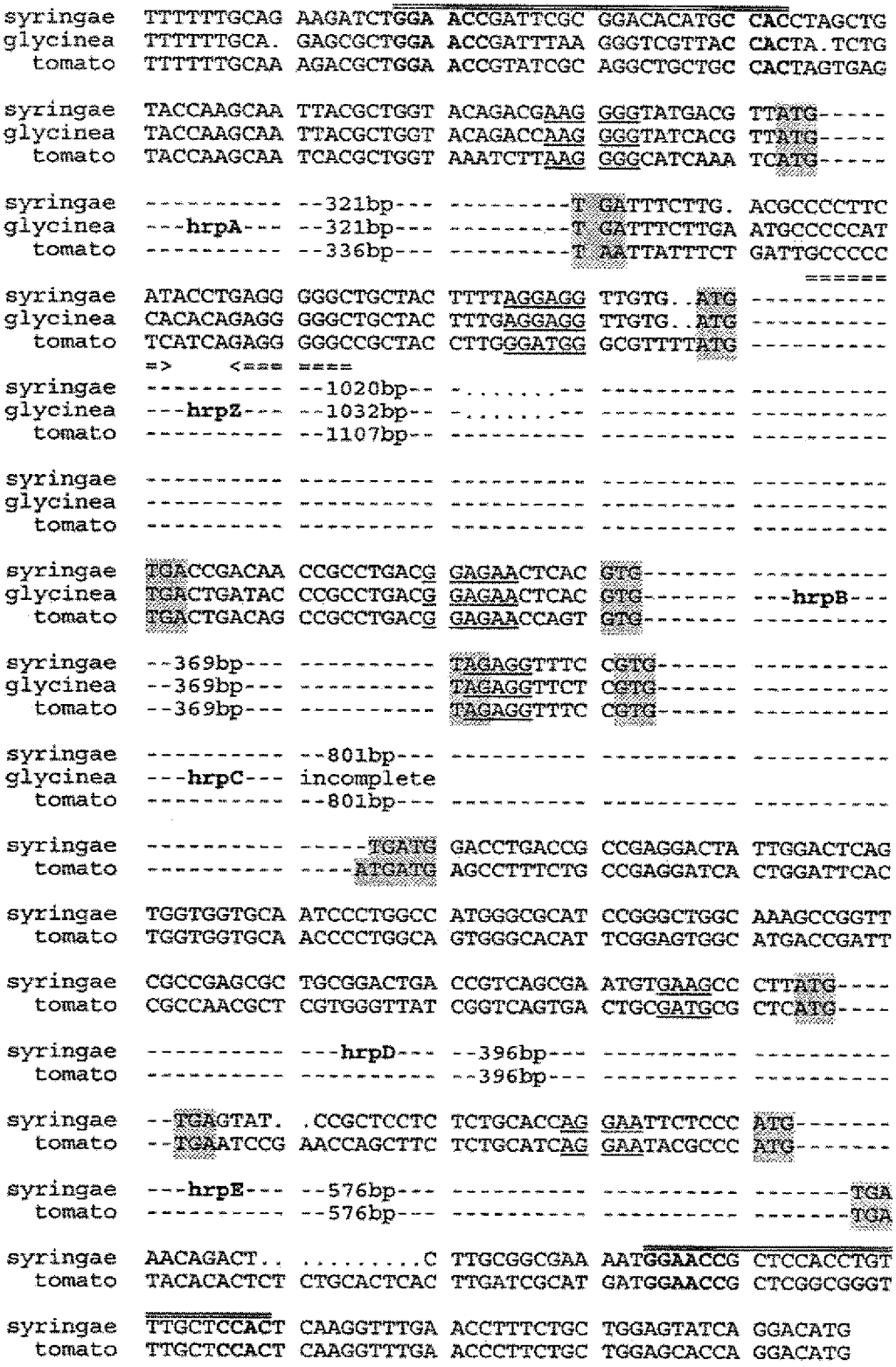

Fig. 3. Nucleotide sequences of the noncoding regions of the *hrpZ* operon from *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato*. The sequences flanking the six ORFs of the *hrpZ* operon were aligned using the PILEUP algorithm (Genetics Computer Group). For *P. s.* pv. *syringae* and *P. s.* pv. *tomato* the sequence extends from immediately downstream of *hrpS* to the end of the operon. For *P. s.* pv. *glycinea* the sequenced region terminates at the beginning of *hrpC*. The proposed initiation and termination codons are highlighted for each ORF. The *hrp/avr* consensus sequences upstream of *hrpA* and *hrpF* are marked by double lines, with the conserved nucleotides in bold and the putative ribosome binding sites for each ORF underlined. A short inverted repeat upstream of *hrpZ* is also indicated with dashed arrows.

al. 1990) did not find significant homology to any other bacterial proteins, with the exception of a single, glycine rich region found only in $HrpZ_{Psy}$ (Fig. 4). This stretch of 24 amino acids has homology at both the nucleotide and amino acid level to a region of the host-specific elicitor PopA1 from *P. solanacearum*, as shown at the bottom of Figure 4. There is no overall similarity of the amino acid and nucleotide sequences of HrpZ to the HR elicitors characterized from *E. amylovora*, *E. chrysanthemi*, and *P. solanacearum* except to a degree accounted for by their similar composition.

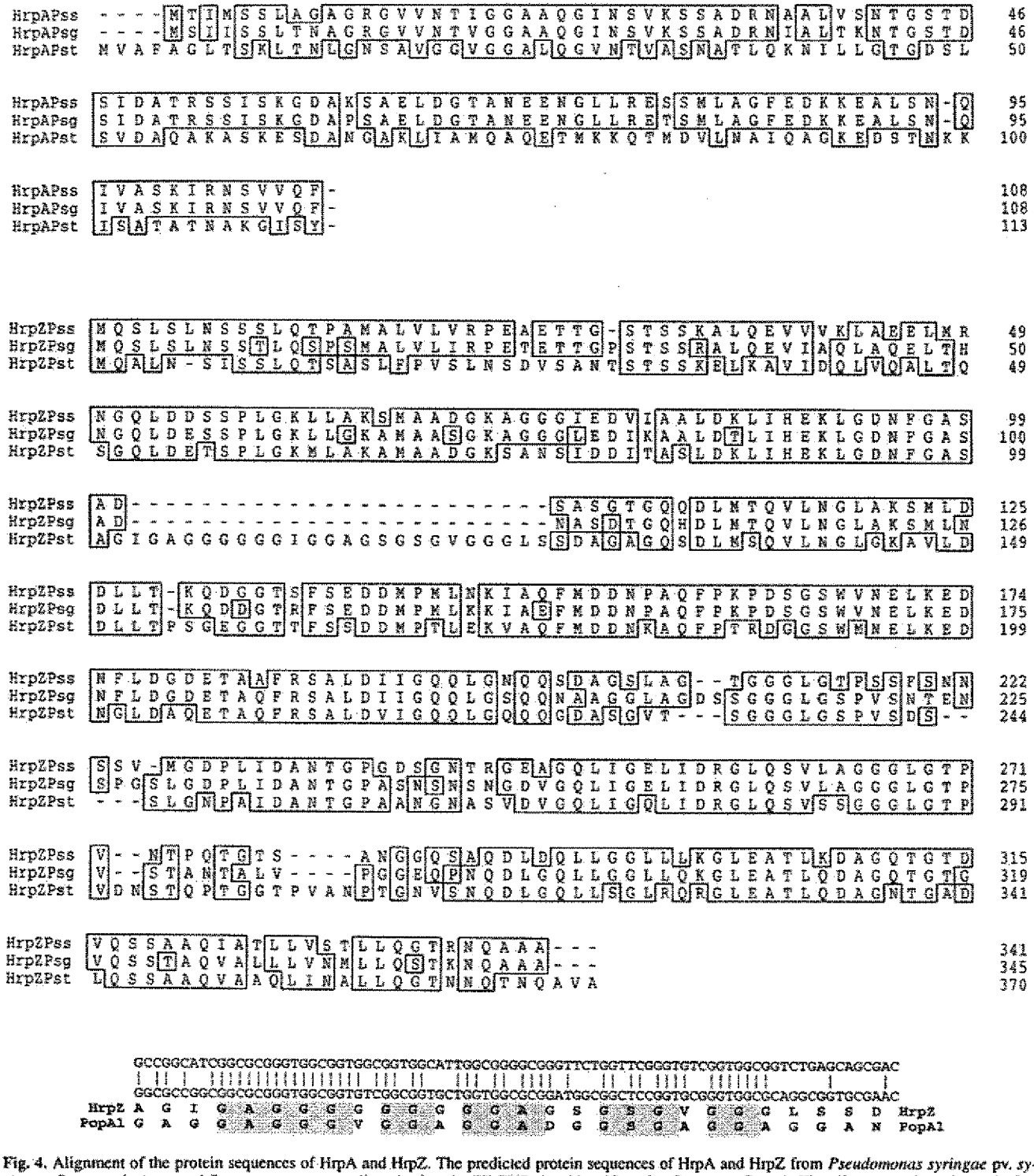

Fig. 4. Alignment of the protein sequences of HrpA and HrpZ. The predicted protein sequences of HrpA and HrpZ from *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato* were aligned using the PILEUP algorithm (Genetics Computer Group). The alignment of a unique glycine rich region of $HrpZ_{Psy}$ with a homologous region of PopA1 from *P. solanacearum* is also shown.

The predicted HrpA protein of *P. s.* pv. *tomato* differs substantially from that of *P. s.* pv. *syringae* and *P. s.* pv. *glycinea*.

The first ORF of the *hrpZ* operon starts 50 bp downstream of the conserved *hrp/avr* promoter motif, as shown in Figure 3. The predicted product is a small (11 kDa), hydrophilic protein with a hydrophobic N-terminus. An alignment of the amino acid sequences from all three pathovars is shown in Figure 4. Although the predicted sequences of HrpA from *P. s.* pv. *syringae* and *P. s.* pv. *glycinea* are highly conserved, with 92% similarity and 91% identity to each other, HrpA from *P. s.* pv. *tomato* is quite divergent, having only 42% similarity and 28% identity to HrpA from *P. s.* pv. *syringae* The presence of a ribosome binding site and the highly conserved character of HrpA in two of the three pathovars supports the hypothesis that HrpA is translated. T7 polymerase-dependent expression of *hrpA* (described below) provides further evidence for production of a HrpA protein. Cell lysates of *E. coli* expressing only HrpA did not elicit the HR on tobacco (data not shown), which suggests that it does not contribute directly to the HR. The role of HrpA in the bacterium is unknown, and it shows no significant homology to any previously characterized proteins.

T7 expression studies.

To confirm the production of proteins corresponding to the two sets of newly cloned *hrpA* and *hrpZ* genes, the *Bgl*II-*Pst*I fragments from *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* were subcloned into pET21(+) and the products specifically labelled by T7 promoter/polymerase-dependent expression in *E. coli* BL21(DE3) cells incubated with [^{35}S]-methionine (Studier et al. 1990). Radiolabeled proteins in the cell lysate were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 5). Lysates of cells containing pCPP2211 displayed unique bands which corresponded well with the predicted molecular weight of HrpA (11.5 kDa) and were consistent with the previously observed mobility of $HrpZ_{Pst}$ (Fig. 5, lane 2). Lysates of cells containing pCPP2210 contained bands corresponding to $HrpZ_{Psg}$ (36 kDa) and HrpA (11 kDa)(Fig. 5, lane 3). No HrpB band was visible in the products of pCPP2211 (Fig. 5, lane 2), but this could potentially be attributed to the omission of cysteine, which is not required for HrpA and HrpZ synthesis, from the amino-acids added to the reaction mixture. T7 expression of HrpB was independently confirmed for both *P. s.* pv. *syringae* and *P. s.* pv. *tomato* using a 0.84-kb *Pst*I-*Age*I fragment of pHIR11 and the 3.7-kb *Sac*I-*Eco*RI fragment from pCPP2209, subcloned into LITMUS 28 to construct the plasmids pCPP2303 and pCPP2304. T7 expression in *E. coli* BL21(DE3) cells was performed as outlined above and in Figure 5. In each case a protein of about 13 kDa was observed, which corresponds well with the predicted molecular weight of HrpB from each of the two pathovars (data not shown). In an accompanying study Huang et al. (1995) have confirmed the production of proteins corresponding to HrpC, HrpD, and HrpE from *P. s.* pv. *syringae* 61. The similarities between the three pathovars suggest that the equivalent ORFs in *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* also encode proteins. However when we independently confirmed the production of HrpD from *P. s.* pv. *syringae* 61 using a 1.3-kb *Sal*I-*Sac*I subclone from pHIR11 cloned into pT7-6 (pCPP2305) our results suggested the use of an alternative initiation codon to make a larger (21 kDa) HrpD protein (data not shown). In the absence of a strong ribosome binding site at either of the putative initiation codons, the exact size of HrpD remains uncertain.

The four ORFs downstream of *hrpZ* show varying similarities to *Yersinia* Ysc proteins.

The *hrpC*, *hrpD*, and *hrpE* genes downstream of *hrpZ* in *P. s.* pv. *syringae* 61 have been sequenced and the products identified using T7 polymerase-dependent expression (Huang et al. 1995). Two of the predicted proteins, HrpC and HrpE, were shown to be homologous to the proteins YscJ and YscL, respectively, which are encoded in the *virC* operon of *Yersinia enterocolitica* and are involved in the type III secretion pathway (Michiels et al. 1991). Homologs of YscJ have also been found in the *hrp* clusters of several other phytopathogenic bacteria, including *P. solanacearum* and *X. campestris* (Fenselau et al. 1992; Gough et al. 1992). Additional homologs are *Salmonella typhimurium* FliF and *Rhizobium fredii* NolT (Jones et al. 1989; Meinhardt et al. 1993). The same four downstream ORFs are found in *P. s.* pv. *tomato*, and the partial sequence of the operon from *P. s.* pv. *glycinea* confirms the presence of the first two of these ORFs, *hrpB* and *hrpC*, in this pathovar (Fig. 6).

HrpB is fairly conserved in all three pathovars, as shown by the alignment presented in Figure 6. It encodes a small serine-rich protein of approximately 13 kDa. BLAST searches using HrpB from either *P. s.* pv. *syringae* or *P. s.* pv. *glycinea* identified no significant homologies, but a search using HrpB from *P. s.* pv. *tomato* identified similarity to the *Yersinia* protein, YscI. YscI is 115 amino acids long, thus slightly shorter than HrpB (127 amino acids). *yscI* lies immediately upstream of *yscJ* in the *virC* operon, which suggests that the downstream ORFs of the *hrpZ* operon might be colinear with a region of the *virC* operon.

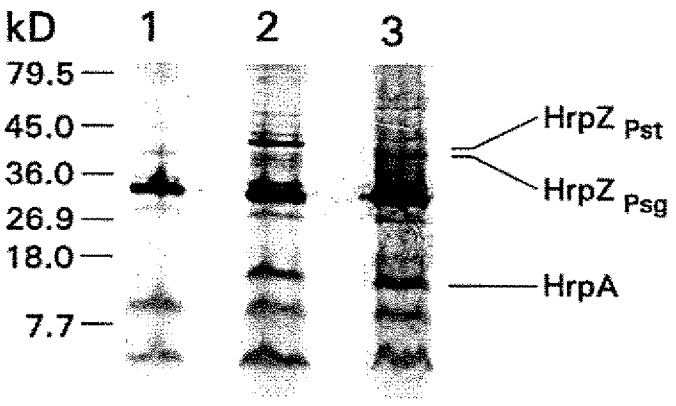

Fig. 5. T7 polymerase-dependent expression and radiolabeling of HrpA and HrpZ. T7 promoter/polymerase expression was carried out using the pET21(+) vector system in *E. coli* BL21(DE3). Cells were grown in LM to an OD_{600} of 0.5, then centrifuged and resuspended in M9 minimal medium supplemented with 0.01% amino acids (lacking methionine and cysteine), glucose and thiamine. Cells were incubated at 30°C for 3 h and then induced with 1 mM IPTG for 10 min, followed by incubation with rifampicin at 300 µg/ml for 30 min. Cells were incubated with 10 µCi [^{35}S]-methionine for 10 min, lysed in SDS-loading buffer, and the proteins were separated by SDS-polyacrylamide electrophoresis and visualized by autoradiography. *E. coli* BL21(DE3) cells carried the following plasmids in lanes: 1, pET21(+); 2, pCPP2211; 3, pCPP2210.

The apparent colinear arrangement of this group of *hrp* and *ysc* genes led us to inspect the *P. s.* pv. *syringae* and *P. s.* pv. *tomato* HrpD proteins for possible similarity to the *Yersinia* spp. YscK proteins. The similarity between the HrpD of *P. s.* pv. *syringae* and *Y. pseudotuberculosis* was the highest, with 28% of the amino acids identical and 57% similar. The HrpD and YscK proteins are of similar overall composition, and they lack any predicted transmembrane segments. However, there is a striking discrepancy between the sizes of the two proteins. HrpD is only 133 amino-acids long, whereas YscK from *Y. pseudotuberculosis* is 209 amino-acids long. From the T7 experiments described above it is important to note that in the absence of a strong ribosome binding site, the precise initiation codon of the *hrpD* ORF is uncertain; it is conceivable that *hrpD* actually initiates immediately downstream of *hrpC*, at the ATG codon which overlaps the stop codon of *hrpC*, which would yield a predicted protein of 176 amino acids for $HrpZ_{Pss}$ or 175 amino acids for $HrpZ_{Pss}$ in an arrangement similar to that of the *yscJ* and *yscK* ORFs in *Yersinia* spp. However, this codon and all other potential initiation codons upstream of the one we have chosen lack ribosome binding sites, and the pattern of codon usage suggests that the intergenic region is not translated.

Although the similarities between HrpB/YscI, HrpD/YscK, and HrpE/YscL are lower than those involving HrpC/YscJ, the similarities of HrpB/YscI and HrpE/YscL are clearly in-

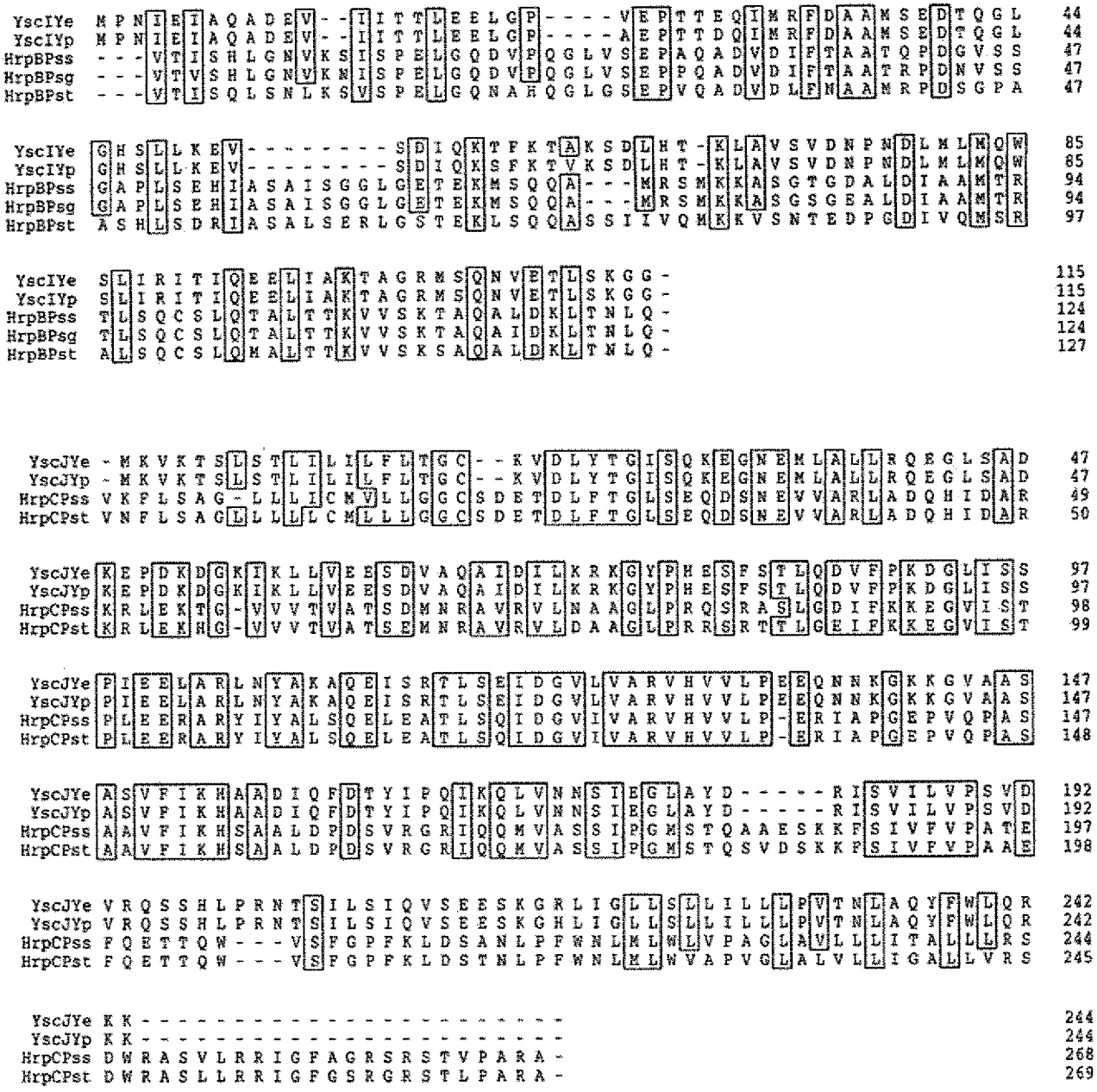

Fig. 6. Alignment of the protein sequences of HrpB from *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato*, and HrpC, HrpD and HrpE from *P. s.* pv. *syringae* and *P. s.* pv. *tomato* with YscI, YscJ, YscK, and YscL from *Y. enterocolitica* and *Y. pseudotuberculosis* (Michiels et al. 1991; Rimpilainen et al. 1992). *(continued on next page)*

dicative of probable homology as based on a difference between the scores for the optimized and the average of 100 random Gap alignments being at least 5 times the standard deviation for the randomized alignments (Doolittle 1986). The scores for HrpD/YscK lie at the margin of significance by this measure. However, the varying levels of similarity are consistent with the divergence observed between Hrp proteins from different *P. syringae* pathovars and between Ysc proteins from different *Yersinia* spp. The results for HrpB,C and E lend support to the weak homology of HrpD to YscK and suggest that *hrpB*, *hrpC*, *hrpD*, and *hrpE* are colinear with *yscI*, *yscJ*, *yscK*, and *yscL*.

In a recent report, Van Gijsegem et al. (1995) observe that the *P. solanacearum* GMI1000 *hrp* cluster also encodes homologs of YscJ and YscL but not YscI and YscK. It is possible that with relatively divergent Hrp sequences, similarities with Ysc proteins may be found only after examining the sequences from several plant pathogens. It is interesting to note that there is no ORF following *hrpE* that is homologous to the protein encoded by the final gene of the *virC* operon, YscM. However, the *hrpZ* operon lies immediately upstream of the *hrpH* operon (Fig. 1), and HrpH is a homolog of YscC, a secretion protein which lies upstream of *yscIJKL* within the *virC* operon (Michiels et al. 1991). This suggests that a sig-

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YscKYe  MMENYITSFQLRFCPAAYLHLEQLPSLWRSILPYLPQWRDSAHLNAALLD   50
YscKYp  MMENYITSFQLRFCPAAYLHLEQLPSLWRSILPYLPQWRDSA--NAALLD   48
HrpDPss ----------------------------------------MVSRHSVFLQ   10
HrpDPst ----------------------------------------MASRHGVFLQ   10

YscKYe  EFSLDTDYEEPHGLGALPLQPQSQLELLLCRLGLVLHGEAIRRCVLASPL  100
YscKYp  EFSLDTDYEEPHGLGALPLQPQSFLELLLCRLGLVLHGEAIRRCVLASPL   98
HrpDPss SIGIT----------PSQPPMPAEPVLNWLALT----------PVQR   37
HrpDPst SLGID----------PAQPPAPAEPVLRWLALT----------PSQR   37

YscKYe  QQLLTLVNQETLRQIIVQHELLIGPWPTNWQRPLPTEIESRTMIQSGLAF  150
YscKYp  --LLTLVNQETLRQIIVQHELLIGPWPTHWQRPLPTEIESRTMIQSGLAF  146
HrpDPss DQALDLAQRICFSR--NESDGHDGQWCWALTKALRPGV--WLELERED AR  83
HrpDPst EQALSLAQCICFSR--NESDGPDGQWCWGLTKALRPGV--WLEFEHED AR  83

YscKYe  WLAAMEPQPQAWCKRLSLRLPLATPSEPWLVAESQRPLAQTLCHKLVKQV  200
YscKYp  WLAAME--PQAWCKRLSLRLPLATPSEPWLVAESQRPLAQTLCHKLVKQV  194
HrpDPss LLLGAWLGPEYWSR---LRLAWAPDEVTDRPCAAPENKLQTLWQAVLWRV  130
HrpDPst LLLGAWLGPQYWSR---LCLECPPNEVPDTPGKAPENKLQALWQAIHWRV  130

YscKYe  MPTCSHLFK                                          209
YscKYp  TPTCSHLFK                                          203
HrpDPss TAT------                                          133
HrpDPst TAA------                                          133

YscLYe  MSQTCQTGYAYMQPFVQIIPSNLSLACGLR--ILRAEDYQSSLTTEELIS   48
YscLYp  MSQTCQTGYAYMQPFVQIIPSNLSLACGLR--ILRAEDYQSSLTTEELIS   48
HrpEPss ----------------MLAKRSITLTADAVLPEPVLRREDIAMSLLARDILT   36
HrpEPst ----------------MLAKRSIALTAATLLREPILRREDIADSLLARDILA   36

YscLYe  AAKQDAEKILADAQEVYEQQKQLGWQAGMDEARTLQATLIHETQLQCQQF   98
YscLYp  AAKQDAEKILADAQEVYEQQKQLGWQAGMDEARTLQATLIHETQLQCQQF   98
HrpEPss DARRQAEQLLVLEQAKADHRHQ----EALAQFWERANAFLDELHVQREAL   82
HrpEPst DARQQPTQILALEQEKAEHLQQ----QALAQFWENANAFLGELQVQREAL   82

YscLYe  YRHVEQQMSEVVLLAVRKILNDYDQVDMTLQVVREALALVSNQKQVVVRV  148
YscLYp  YRHVEQQMSEVVLLAVRKILNDYDQVAMTLQVVREALALVSNQKQVVVRV  148
HrpEPss QQQAMTAVEELLTEALCQLLDETTLAERARALVRNLAASQLNEAVATLSV  132
HrpEPst QEQAMTAVEELLSESLRHLLDDTTLAERARALARNLPSNQLNEAVATLSV  132

YscLYe  NPDQAGTIREQIAKVHKDPPEISYLEVTADARLDQGGCILETEVGIIDAS  198
YscLYp  NPDQAGAIREQIAKVHKDFPEISYLEVTADARLDQGGCILETEVGIIDAS  198
HrpEPss HPEMAEPVAEWLAESR--FAE--HWELKRDATLTTESLRLLSDANGAPEID  178
HrpEPst HPQIADPVAEWLADSR--FSE--HWQLKRDATIASDSLRLLSDANGAPDIA  178

YscLYe  IDGQIEALSRAISTTLGQMKVTEEE                           223
YscLYp  IDGQIEALSRAISTTLGQMKVTE--                           221
HrpEPss WATLRNGLAGAEPAA----------                           193
HrpEPst WADLRKGLLGVEPAA----------                           193
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Fig. 6. *(continued from preceding page)*

nificant proportion of the *virC* operon is conserved in *P. syringae*, albeit in a rearranged form. Eckhardt (1978) gels of total DNA, Southern-blotted and probed with a 0.75-kb *Bst*XI internal fragment of $hrpZ_{Pss}$, suggested that the *hrp* genes are chromosomal in the three strains of *P. syringae* studied, rather than being plasmid-borne as are the *hrp* genes of *P. solanacearum* GMI1000 or the *ysc* genes of *Yersinia* spp. (Van Gijsegem et al. 1993; data not shown). The homologies of the *hrpZ* operons are summarized in Table 1.

Overexpression, purification, and biological assay of $HrpZ_{Pst}$ and $HrpZ_{Psg}$.

Partially purified lysates of *E. coli* expressing $HrpZ_{Pst}$ and $HrpZ_{Psg}$ elicited a clear HR on tobacco while control lysates of *E. coli* containing vector alone did not. However the activity of the cell lysates on the two host plants was more ambiguous. Soybean is generally unreactive to cell lysates from either pathogen, while tomato is quite sensitive and sometimes weakly reactive not only to cell lysates of *E. coli* expressing HrpZ, but also to control lysates of *E. coli* containing vector alone. To accurately evaluate the biological properties of HrpZ from each of the two pathovars, it was necessary to purify HrpZ. It was also necessary to ascertain that the HR observed on tobacco was due solely to HrpZ and not to the products of either of the two flanking ORFs, HrpA and HrpB, since HrpA and a fusion protein of HrpB were being expressed in addition to HrpZ by the original $hrpZ_{Psg}$ and $hrpZ_{Pst}$ clones.

As a first step towards purifying HrpZ, we attempted to increase the level of expression. From the sequence of the *Pst*I clones encoding *hrpZ* it was clear that long stretches of DNA encoding *hrpA* and the 3′ end of *hrpS* (1,144 bp in $hrpZ_{Psg}^{+}$ pCPP2202 and 809 bp in $hrpZ_{Pst}^{+}$ pCPP2203) separated *hrpZ* from the *lac* promoter in pBluescript II. A series of deletions of the 5′ end of the $hrpZ_{Pst}$ clone were constructed using the Erase-a-Base system (Promega), bringing the *lac* promoter within 100 bp of the *hrpZ* initiation codon, and removing *hrpA*. Although cell lysates expressing the deleted clones retained HR eliciting activity, they did not show a substantial increase in gene expression. Searching for an explanation for this behavior we identified a number of potential contributing factors. The first possibility was the presence of a *cis*-acting sequence contained in the 100 bp remaining upstream of $hrpZ_{Pst}$. Using a terminator analysis program we identified a 9-bp inverted repeat located between *hrpA* and *hrpZ* (Fig. 3). Although this repeat lacks the AT-rich sequence downstream which is characteristic of many terminators, it is possible that its presence encourages premature transcription termination. Similar repeats, albeit with weaker secondary structure, can be found upstream of $hrpZ_{Pss}$ and $hrpZ_{Psg}$. A second factor contributing specifically to the low expression of $hrpZ_{Pst}$ may be the absence of a strong ribosome binding site. Finally, there could be factors related to the proteins themselves, such as a lack of stability.

To eliminate possible *cis*-acting sequences and to obtain clones of $hrpZ_{Pst}$ and $hrpZ_{Psg}$ that lack *hrpA* and *hrpB*, the *hrpZ* genes from both pathovars were amplified by PCR, directionally cloned into pBluescript II and transformed into *E. coli* DH5α F′*lacI*^{q}. We obtained significantly increased expression of $HrpZ_{Psg}$ using the plasmid pCPP2255 (Fig. 7), but unexpectedly, overexpression of $HrpZ_{Pst}$ appeared to be deleterious to the cells, and plasmids recovered from transformants often showed rearrangements. To maximize expression of $HrpZ_{Pst}$ under these conditions, we introduced subclones containing the gene behind the T7 promoter of pET21(+) (Novagen, Madison, WI). Unlike the *lac* promoter, the T7 promoter is less sensitive to distance effects, and expression of $HrpZ_{Pst}$ in *E. coli* BL21(DE3), with pET21(+) as the vector, resulted in increased expression as shown in Figures 2 and 8. Expression in BL21(DE3) also allowed us to retain almost complete repression of *hrpZ* until induction with IPTG. Good expression of $HrpZ_{Pst}$ was achieved using the plasmid pCPP2211 in *E. coli* BL21(DE3).

The quality of the samples obtained following partial purification of the lysates by heat treatment was quite variable. To ensure removal of the majority of the contaminating proteins and to obtain a more concentrated sample of protein, we further purified HrpZ by ammonium sulphate precipitation and hydrophobic chromatography, which as indicated in Figure 8, yielded a distinct band on a Coomassie-stained gel. Purified, active HrpZ could then be obtained by electroelution from excised gel slices. This procedure was also used to isolate

Table 1. Homologies of *Pseudomonas syringae* pv. *syringae* *hrpZ* operon proteins with proteins from other *P. syringae* pathovars and *Yersinia* spp.

P. s. pv. *syringae*	HrpA (108)[a]	HrpZ (341)	HrpB (124)	HrpC (268)	HrpD (133)[d]	HrpE (193)
P. s. pv. *glycinea*	(108)	(345)	(124)			
	91/92[b]	79/87	94/96			
P. s. pv. *tomato*	(108)	(370)	(124)	(268)	(133)	(193)
	28/42	63/75	68/80	90/95	78/87	76/87
Y. enterocolitica			YscI	YscJ	YscK	YscL
			(115)	(244)	(203)	(223)
			22/45[c]	35/59	26/53	21/47
			24/45	38/60	22/48	22/46
Y. pseudotuberculosis			(115)	(244)	(209)	(221)
			22/45	35/59	28/57	21/47
			21/44	38/60	23/49	22/46

[a] Number of amino acids in the protein is given in parentheses.
[b] Percent identical and similar amino acids in comparison with the *P. s.* pv. *syringae* protein.
[c] The first pair of values are the percent identical and similar amino acids in comparison with the *P. s.* pv. *syringae* protein; the second are in comparison with *P. s.* pv. *tomato*.
[d] The data presented here are for the shorter of the two potential ORFs encoding *hrpD*. The larger versions of the HrpD proteins of *P. s.* pv. *syringae* and *P. s.* pv. *tomato* would be respectively 175 and 176 amino acids long with 74/84% identity/similarity to each other.

HrpZ from the supernatants of *P. s.* pv. *tomato* and *P. s.* pv *glycinea* grown in *hrp*-inducing minimal media (Fig. 9). Preparations of the purified HrpZ proteins from *P. s.* pvs. *syringae*, *glycinea*, and *tomato*, at a concentration of ≥20 μM in MES buffer, were infiltrated into the leaves of tobacco, soybean, and tomato. The three proteins elicited a collapse involving >50% of the infiltrated tissue in tobacco and tomato leaves that developed within 18 h and was typical of the HR elicited by incompatible *P. syringae* strains, but they caused no visible reaction in soybean. It is worth noting that tobacco and tomato plants vary substantially in their sensitivity to harpin preparations. For example, some leaves on sensitive tomato plants will respond to 2 to 5 μM $HrpZ_{Pst}$, but ≥20 μM is required for consistent results. Furthermore, unlike tobacco, tomato plants that have responded hypersensitively to a HrpZ preparation do not respond to subsequent infiltrations of the elicitor. The spurious necroses sometimes observed were deduced to result from mechanical damage incurred during infiltration or the infiltration of preparations contaminated with salts or containing high concentrations of vector control *E. coli* lysates. These necroses developed much more quickly (within 4 to 6 h), and were much weaker and patchier than the confluent HR elicited by HrpZ. The fact that the HR induced by HrpZ in tomato and tobacco is an active response of host tissue was confirmed by coinfiltration of either sodium vanadate at $5^{-5} \times 10^{-3}$ M or lanthanum chloride at 1×10 M. Each of these two inhibitors of plant metabolism completely inhibited the HR elicited by HrpZ preparations from each of the three pathovars but not the necrosis caused by the other factors mentioned.

DISCUSSION

We have used the *P. s.* pv. *syringae* 61 *hrpZ* gene to isolate the *hrpZ* locus from *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000. Characterization of the *hrpZ* genes, products, and flanking DNA of these three pathovars has revealed the structure of the *hrpZ* operon, the relative variation among ORFs within the operon, the presence of genes downstream of *hrpZ* that are colinear with a block of genes involved with *Yersinia* virulence protein secretion, and the presence in $HrpZ_{Pst}$ of a sequence related to a sequence in the PopA1

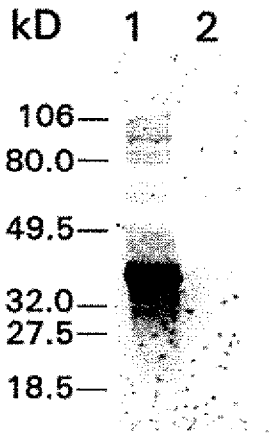

Fig. 7. Overexpression of $HrpZ_{Pst}$ in *E. coli* DH5α F'*lacI*^{Q}. Cultures were grown overnight at 30°C in LM with 1 mM IPTG. Cell lysates were partially purified by heat treatment, separated on an SDS-polyacrylamide gel, transferred to Immobilon-P, immunoblotted with anti-$HrpZ_{Pst}$ antibodies, and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase. Lanes: 1, *E. coli* DH5α F'*lacI*^{Q} (pCPP2255); 2 *E. coli* DH5α F'*lacI*^{Q} (pBluescript II).

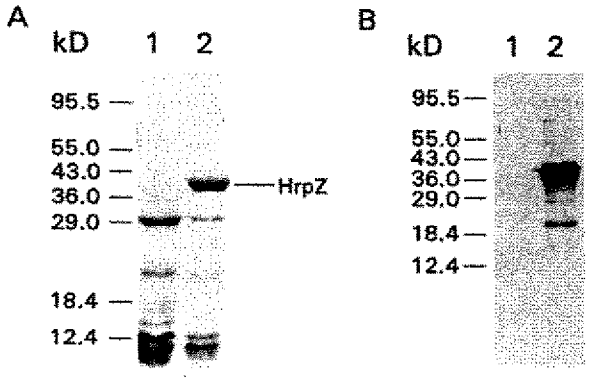

Fig. 8. Overexpression and purification of $HrpZ_{Pst}$. Cultures were grown to an OD_{600} of 0.6 and induced with 1 mM IPTG. $HrpZ_{Pst}$ was then partially purified from the cell lysate in a three-step process: first, by heat-treatment at 100°C as previously described, then by precipitation with ammonium sulphate at 30 to 45% saturation, and finally by binding to a hydrophobic resin (phenyl-sepharose) at 30% ammonium sulphate. **A**, Coomassie stained SDS-polyacrylamide gel. Lanes: 1, *E. coli* BL21(DE3)(pET21+); 2, *E. coli* BL21(DE3)(pCPP2211). **B**, Immunoblot of the samples shown in A, probed with anti-$HrpZ_{Pst}$ antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase.

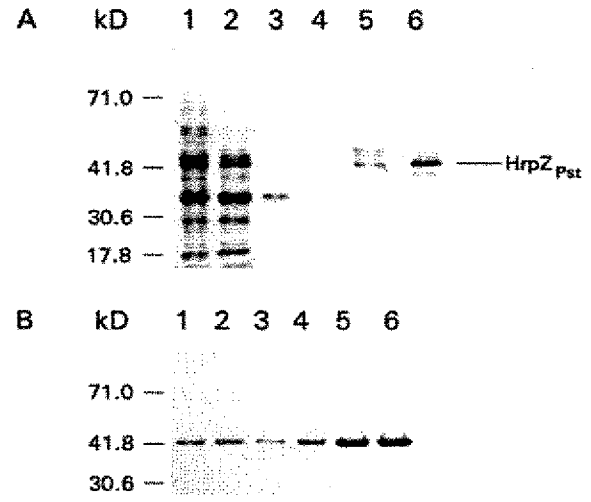

Fig. 9. Purification of $HrpZ_{Pst}$ from *hrp*-induced *Pseudomonas syringae* pv. *tomato*. Cells were grown in King's broth (KB) at 30°C and then resuspended in *hrp*-inducing minimal medium (Huynh et al. 1989) and incubated at room temperature overnight. Cells were removed by centrifugation and the supernatant heat-treated at 100°C for 10 min. Proteins in the supernatant were precipitated with ammonium sulphate at the percent saturations indicated. Proteins were desalted, concentrated, and resuspended in 5 mM MES using Centricon-10 tubes (Amicon). **A**, Coomassie stained SDS-polyacrylamide gel. Lanes: 1, supernatant extracted with Strataclean resin (Stratagene); 2, heat-treated supernatant extracted with Strataclean resin (Stratagene); 3, 0 to 20% ammonium sulphate fraction; 4, 20 to 30% ammonium sulphate; 5, 30 to 40% ammonium sulphate; 6, 30 to 45% ammonium sulphate. **B**, Immunoblot of the samples shown in A, probed with anti-$HrpZ_{Pst}$ antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase.

protein of the tomato pathogen *P. solanacearum* GMI1000. We also observed that purified $HrpZ_{Pst}$ was at least as effective as $HrpZ_{Pss}$ and $HrpZ_{Psg}$ in eliciting an HR-like necrosis in the leaves of tomato, a host of *P. s.* pv. *tomato* DC3000, whereas none of the HrpZ preparations elicited significant necrosis in soybean, the host of *P. s.* pv. *glycinea*.

The HrpZ proteins of three *P. syringae* pathovars.

A comparison of the sequences of the three HrpZ proteins with each other and with HR elicitors characterized from other bacteria indicates that the HrpZ proteins represent a distinct family of elicitors that is conserved among *P. syringae* pathovars. The amino acid sequences of the three proteins are sufficiently similar to reveal their relatedness, but (with the exception of a sequence within $HrpZ_{Pst}$), they show no significant relatedness to elicitor proteins from other bacteria. Interestingly, *hrpZ* is the second most divergent ORF in the *hrpZ* operons of *P. s.* pv. *syringae* and *P. s.* pv. *tomato*, with only 63% of the predicted amino acids being identical. Nevertheless, $HrpZ_{Pss}$, $HrpZ_{Psg}$, and $HrpZ_{Pst}$ are indistinguishable in several biological and physical properties. They have the same effect on different plants (discussed below), and they are heat stable, glycine-rich, and devoid of cysteine and tyrosine. The lack of tyrosine is a feature they differentially share with the *P. solanacearum* PopA1 protein but not the *Erwinia* harpins. This property has been speculated to allow the protein to avoid the H_2O_2-mediated cross-linking of tyrosine residues that may occur in plant cell walls during defense responses (Bradley et al. 1992; He et al. 1993).

Interestingly, a 24 amino acid, glycine-rich stretch of $HrpZ_{Pst}$ shows homology to part of PopA1, as does the cognate nucleotide sequence. The region of homology between $HrpZ_{Pst}$ and PopA1 corresponds exactly to the insertion in $HrpZ_{Pst}$. The insertion of this element within $HrpZ_{Pst}$ sequences that are otherwise similar among the three HrpZ proteins suggests horizontal transfer and a common ancestry with PopA1. Because the host range of *P. solanacearum* overlaps with that of *P. s.* pv. *tomato*, it is tempting to speculate that this region has some particular significance to pathogenesis on tomato, although, as discussed below, this is not obvious from the different effects of the two proteins on tomato.

The presence of this insert in active $HrpZ_{Pst}$ is another indicator of the apparent plasticity of structure/function relationships in these glycine-rich elicitor proteins. That significant changes to the structure of these proteins does not abolish their activity was previously demonstrated when a fortuitous $hrpZ_{Pss}$ clone was found to produce an active derivative of HrpZ missing the N-terminal 125 amino acids, and the *popA* product was observed to be degraded in culture to an active form missing the N-terminal 93 amino acids (He et al. 1993; Arlat et al. 1994). Clearly the presence of this "additional" internal sequence does not diminish the ability of the protein to elicit the HR. In fact, although it is difficult to make a quantitative assessment, $HrpZ_{Pst}$ may actually be a slightly more potent elicitor of the HR than $HrpZ_{Pss}$.

However, $HrpZ_{Pst}$ appears to differ from the other HrpZ proteins in being deleterious to *E. coli* cells when overexpressed and is possibly more unstable, making it difficult to purify large amounts of the protein. Since the glycine-rich region is the most obvious difference between $HrpZ_{Pst}$ and $HrpZ_{Pss}$ it is possible that it contributes to this phenomenon. We were able to overcome this problem experimentally by using a tightly regulated T7 promoter/polymerase system, but never obtained quite the same level of expression we achieved with $HrpZ_{Pss}$ and $HrpZ_{Psg}$. However, there remains the obvious question of how HrpZ toxicity is avoided by *P. s.* pv. *tomato*. One possibility would be that HrpZ is never expressed at levels high enough to affect the bacterium, even when it is induced in planta. Some indirect evidence for this hypothesis is provided by our examination of the DNA upstream of $hrpZ_{Pst}$. The ORF has a weak ribosome binding site, and we also observed that expression of cloned *hrpZ* from the *lac* promoter appears to be attenuated by the presence of *cis*-acting upstream sequences. A 9-bp GC-rich repeat upstream of *hrpZ* may be significant in this regard. Preliminary data from northern blotting experiments also indicate that premature transcription termination may take place when *hrpA-hrpZ* clones are expressed in *E. coli* (G. Preston, unpublished). A second possibility is that the location of the *hrpZ* gene in an operon with secretion genes ensures tight coupling of synthesis and secretion. Genes encoding extracellular proteins and secretion pathway components are often coregulated, but with a few exceptions involving the type I pathway, they do not lie within the same operon (Fath and Kolter 1993). A third possibility is that *P. s.* pv. *tomato* is more tolerant of high levels of HrpZ than is *E. coli*, or it possesses a means of keeping HrpZ in a nontoxic form while it is in the cell.

Further comparison with the *Yersinia* virulence system presents an intriguing possibility in this regard. It has been shown that secretion of certain "Yops" (the *Yersinia* pathogenicity determinants), involves chaperone proteins, small hydrophilic proteins which help keep the Yop protein in a translocation competent form and help target it for secretion (Wattiau et al. 1994). The genes encoding each chaperone are located adjacent to the gene encoding the corresponding Yop. Given the presence of several small ORFs of undetermined function in the pHIR11 *hrp* cluster, it is tempting to speculate that one of them, particularly *hrpA*, might encode a protein with chaperone function. There is a superficial resemblance between HrpA and *Yersinia* chaperones such as SycE. They are all small, hydrophilic, cytoplasmic proteins which lack a signal sequence, but there are no specific homologies. We are now constructing nonpolar mutations to test the role of HrpA in secretion. Preliminary results suggest that HrpA is not required for *E. coli* MC4100(pHIR11) to elicit an HR or secrete HrpZ (J. R. Alfano, unpublished), but in chaperone-mediated systems limited secretion of a protein will usually occur even in the absence of its chaperone, so it may be necessary to look quantitatively at secretion and accumulation of HrpZ to assess whether mutations in *hrpA* or other *hrp* genes have an effect.

The colinear relationship between several *hrp* and *ysc* genes.

From the sequence of the *hrpZ* operon it is clear that the parallels with the *Yersinia* type III secretion pathway extend beyond homologies of individual genes. The four genes downstream of *hrpZ*, *hrpB–E*, appear to be arranged colinearly with the region of the *virC* secretion operon from *Yersinia* that encodes YscI–L. The *virC* operon is a large operon containing 13 genes, *yscA-yscM*, several of which have been demonstrated to have a role in Yop secretion (Michiels et al. 1991). Of the four *Yersinia* genes with putative ho-

mologs in the *hrpZ* operon, only *yscJ* and *yscL* are known to have a role in secretion. An accompanying paper shows that five more *hrp* genes, downstream of the *hrpH* operon, are colinear with the *yscQ–U* genes in the *virB* operon of *Yersinia* (Huang et al. 1995).

It appears that a significant proportion of the type III secretion pathway described in *Yersinia* can be identified in *P. syringae*, and it seems likely that increasing parallels between the two systems will be found. In both systems the secreted proteins are involved with early events in the interaction with the host, and expression of secretion genes and virulence proteins is tightly coregulated. The secretion pathway seems to function in a similar way, as in both cases secreted proteins lack an N-terminal signal peptide and are not posttranslationally processed.

HrpZ and host specificity.

The function of HrpZ in compatible interactions is unclear. A likely role is the release of nutrients to the apoplast. Atkinson and Baker (1987a, 1987b) have proposed that the alkalinization of the apoplast caused by Hrp$^+$ bacteria (which occurs at a slower rate in compatible interactions) results in the leakage of sucrose and other nutrients to support bacterial growth. One of the key unanswered questions regarding the *P. syringae* HrpZ proteins is their role in host specificity. Compatible interactions leading to disease are distinguished by the absence of the HR. Host-differential elicitor activity would be one way to reconcile the production of HR-eliciting proteins by *P. syringae* and the phenomenon of host-specific compatibility. The failure of the PopA1 protein to elicit the HR in tomato, a host of *P. solanacearum* GMI1000, supports this concept (Arlat et al. 1994). Similarly, the isolated *P. s.* pv. *syringae* 61 HrpZ protein fails to elicit the HR in bean, although the significance of this is diminished by the fact that bean leaves appear insensitive to any harpins (He et al. 1993). To further explore this question, we infiltrated all three HrpZ proteins into the leaves of the host plants for each of the pathovars. The host plants of *P. s.* pv. *syringae* 61, and *P. s.* pv. *glycinea*, bean and soybean, respectively, are uniformly unreactive to HrpZ from both compatible and incompatible pathogens; however, tomato leaves proved to be highly sensitive to all three HrpZ proteins. Thus, our data argue against the hypothesis that host-differential activity of HrpZ proteins controls the host specificity of *P. syringae* pathovars.

If isolated $HrpZ_{Psi}$ elicits the HR in tomato, why does *P. s.* pv. *tomato* not elicit the HR during pathogenesis? One possibility is that the response of tomato to $HrpZ_{Psi}$ is qualitatively different than the response to $HrpZ_{Pss}$ and $HrpZ_{Psg}$ despite manifestation of the same gross morphology. That is, the necrosis elicited by $HrpZ_{Psi}$ is fundamentally different than the HR and does not involve associated defenses that stop the pathogen. We are now testing this possibility with probes for HR-specific transcripts. A second possibility is that $HrpZ_{Psi}$ production is regulated in a host-specific manner. However, *hrpZ* is clearly part of the Hrp regulon: *hrpZ* expression is transcriptionally linked with genes encoding components of the secretion pathway, the *hrpZ* operons in all three of these *P. syringae* pathovars have virtually the same *hrp/avr* promoter sequence, and expression of the *hrpZ* operon is likely required for pathogenicity. The conserved promoter sequences suggests that the *hrpZ* operon is regulated in *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* by the same nutritional conditions and HrpR, HrpS, HrpL regulatory cascade described for *P. s.* pv. *syringae* and *P. s.* pv. *phaseolicola* (Grimm and Panopoulos 1989; Rahme et al. 1992; Xiao et al. 1992; Xiao et al. 1994; Xiao and Hutcheson 1994; Grimm et al. 1995). Whether differential expression of the Hrp regulon controls host specificity awaits determination. A third possibility is that the *P. syringae* pathovars produce host-specific suppressors of defense responses. This is supported by the observation that compatible pathogens do not trigger defense responses in host plants that are elicited by nonpathogens (Jakobek et al. 1993).

It is important to note that our data do not eliminate the possibility that the three HrpZ proteins actually have differential activity in host plants when delivered by living bacteria and that the HR observed may be an abnormal response resulting from the presentation of a high concentration of HrpZ in an artificial manner. In that regard, it is interesting that legumes, which appear insensitive to isolated harpins, respond to Hrp recombinant *E. coli* cells that secrete the same proteins (He et al. 1993). Experiments in which the *hrpZ* genes of *P. syringae* pathovars are switched or altered in their patterns of deployment should test more definitively the role of HrpZ in determining host specificity.

In conclusion, we have characterized an operon containing two components of the Hrp$^+$ system of *P. syringae*—a block of secretion-related genes that are conserved in eukaryotic pathogens in the genera *Pseudomonas*, *Xanthononas*, *Erwinia*, *Yersina*, *Shigella*, and *Salmonella* and a gene encoding an elicitor that is unique to plant pathogens. The elicitors found in the *P. syringae* pathovars are a subfamily of a larger class that appears to be characteristic of plant pathogens, and which we postulate to have a role in releasing nutrients for bacterial utilization. Our challenge now is to determine how the various components of the Hrp system have been adapted to serve plant parasitism in the face of plant defenses.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacteria and plasmids used in this study are shown in Table 2.

Pseudomonads were routinely grown in King's B broth (King et al. 1954) at 30°C, but for certain experiments the *hrp*-derepressing minimal medium of Huynh et al. (1989), adjusted to pH 5.5, was used. *E. coli* was grown in LM (Sambrook et al. 1989) or terrific broth (Tartof and Hobbs 1987). Plasmids were introduced into bacteria by transformation (Sambrook et al. 1989) or electroporation (Gene Pulser, Bio-Rad).

Plant materials.

The plants used in this study were tobacco (*Nicotiana tabacum* L. 'Xanthii'), tomato (*Lycopersicon esculentum* Mill. 'Moneymaker'), and soybean (*Glycine max* L. 'Harosoy'). Plants were grown in a greenhouse or growth chamber at 23° to 25°C with a photoperiod of 16 to 24 h. Infiltration of plant leaves with HrpZ preparations was performed with blunt syringes as described (Huang et al. 1988).

DNA analysis and sequencing.

All DNA manipulations, except where specified, followed standard protocols (Ausubel et al. 1987; Sambrook et al. 1989). The *hrpZ* region of pHIR11 was subcloned into

pBluescript II (Huang et al. 1995). Two *Pst*I fragments of 2.2 and 2.4 kb from pCPP2201 and pCPP2200, respectively, were subcloned into pBluescript II SK(–) in both orientations. A series of overlapping nested deletions covering both strands was generated for each of the subclones using Erase-a-Base (Promega, Madison, WI). The deletions were sequenced from double-stranded templates using Sequenase version 2.0 (U.S. Biochemicals, Cleveland, OH) and forward and reverse M13 primers. Sequencing was completed using specific primers synthesized by Integrated DNA Technologies (Coralville, IA). In addition, the 3.7 and 3.6 kb *Sac*I-*Eco*RI fragments, which overlap the *Pst*I subclones from pCPP2201 and pCPP2200, were also subcloned into pBluescript II SK(–) and sequenced using the ABI 373A DNA sequencer at the Cornell Biotechnology Program DNA sequencing facility and specific primers synthesized by IDT. Nucleotide and derived amino acid sequences were analyzed with the Genetics Computer Group Sequence Analysis Software Package (Devereaux et al. 1984). Homology searches against major sequence databases were done with the BLAST program (Altschul et al. 1990).

PCR amplification of *hrpZ* from *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*.

The *hrpZ* genes of *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* were amplified by PCR from the plasmids pCPP2202 and pCPP2203, respectively. Reactions were performed using the PCR Optimizer kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Reactions were overlaid with mineral oil and incubated in a Hybaid Thermal Reactor (Hybaid, Teddington, U.K.) using these cycle parameters: 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 2 min at 55°C, 3 min at 72°C, followed by a final incubation of 7 min at 72°C. The primers used for $hrpZ_{Psg}$ were 5′-TACGGGATCCTTTGAGGAGGTTGTGATG-3′ and 5′-TACGCTGCAGTATC AGTCAGGCAGCAGC-3′, and those for $hrpZ_{Pst}$ were 5′-TACGGGATCCATGCAAGCACTTA ACAGC-3′ and 5′-GGAACTGCAGCAAGCTCCGGCGA-TACAC-3′. All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and were designed to introduce a *Bam*HI and a *Pst*I site at the 5′ and 3′ ends, respectively, of each amplified fragment.

The $hrpZ_{Psg}$ fragment from pCPP2202 was successfully amplified in all reaction buffers tested. The $hrpZ_{Pst}$ fragment from pCPP2203 was successfully amplified using reaction buffer B (reaction concentration 60 mM Tris-HCl, 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, pH 8.5). PCR products of the expected sizes of 1.0 and 1.2 kb were purified from an agarose gel, digested with *Pst*I and *Bam*HI, cloned into pBluescript II, and then transformed into *E. coli* DH5α F′*lac*I, yielding plasmid pCPP2255 carrying $hrpZ_{Psg}$. Plasmids containing

Table 2. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Reference or source
Escherichia coli		
DH5α	*sup*E44 Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 Nal^r	Hanahan 1983; Life Technologies, Inc. Grand Island, NY
DH5α F′*lacI*^{q}	F′ *pro*AB$^+$ *lacI*^{q}ZΔM15 zzf::Tn5[Km^r]/ϕ80d *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *end*A1 *rec*A1 *hsd*R17 ($r_k^-m_k^+$) *deoR thi*-1 *sup*E44λ$^-$ *gyr*A96 *rel*A1	Life Technologies Inc.
BL21(DE3)	F$^-$ *ompT hsd*B_B ($r_B^-m_B^-$) *dcm gal* DE3	Novagen
Pseudomonas syringae		
pv. *syringae* 61	Wild type	Baker *et al.* 1987
pv. *glycinea* race 4	Wild type	C. J. Baker
pv. *tomato* DC3000	Wild type, Rp^r	D. E. Cuppels
Plasmids		
pBluescript II SK(-)	Cloning vector, Amp^r	Stratagene
pUCP19	pUC19 derivative, Amp^r	Schweizer 1991
pET21(+)	T7 transcription vector, Amp^r	Novagen
pT7-6	T7 transcription vector, Amp^r	Tabor and Richardson 1988
LITMUS 28	Cloning vector, Amp^r	New England Biolabs
pHIR11	25-kb cosmid containing *P.s.* pv. *syringae* 61 *hrp* cluster	Huang *et al.* 1988
pSYH10	$hrpZ_{Pst}$ ORF in pBluescript II	He *et al.* 1993
pCPP2303	0.8-kb *Pst*I-*Age*I subclone from pHIR11, containing *hrpB*, in LITMUS 28	This study
pCPP2305	1.3-kb *Sal*I-*Sac*I subclone from pHIR11, containing *hrpD*, in pT7-6	This study
pCPP2200	pUCP19 carrying 10-kb partial *Sau*3A1 fragment of *P. s.* pv. *glycinea* DNA with $hrpZ_{Psg}$	This study
pCPP2202	2.4-kb *Pst*I subclone of pCPP2200 in pBluescript II; $hrpA_{Psg}$ and $hrpZ_{Psg}$ in expressed orientation with respect to P_{lac}	This study
pCPP2204	As pCPP2202 but with $hrpZ_{Psg}$ in reversed orientation to P_{lac}	This study
pCPP2206	2.4-kb *Pst*I $hrpA_{Psg}$ and $hrpZ_{Psg}$ subclone from pCPP2202 in pET21(+)	This study
pCPP2208	3.6-kb *Sac*I-*Eco*RI $hrpZ_{Psg}$ subclone from pCPP2200 in pBluescript II	This study
pCPP2210	1.85-kb *Bgl*II-*Pst*I $hrpZ_{Psg}$ subclone from pCPP2202 in pET21(+)	This study
pCPP2255	PCR-amplified $hrpZ_{Psg}$ ORF in pBluescript II	This study
pCPP2201	pUCP19 carrying 10-kb fragment of *P. s.* pv. *tomato* DNA with $hrpZ_{Pst}$	This study
pCPP2203	2.2-kb *Pst*I subclone of pCPP2201 in pBluescript II; $hrpA_{Pst}$ and $hrpZ_{Pst}$ in expressed orientation with respect to P_{lac}	This study
pCPP2205	As pCPP2203 but with $hrpZ_{Pst}$ in reversed orientation to P_{lac}	This study
pCPP2207	2.2-kb $hrpZ_{Pst}$ subclone from pCPP2203 in pET21(+)	This study
pCPP2209	3.7-kb *Sac*I-*Eco*RI subclone from pCPP2201 containing $hrpBCDE_{Pst}$ in pBluescript II	This study
pCPP2304	3.7-kb *Sac*I-*Eco*RI subclone from pCPP2209 in LITMUS 28	This study
pCPP2211	2.0-kb *Bgl*II-*Pst*I $hrpZ_{Pst}$ subclone from pCPP2203 in pET21(+)	This study

[a] Amp^r = ampicillin resistance; Nal^r = nalidixic acid resistance; Rp^r = rifampicin resistance.

PCR-amplified $hrpZ_{Pst}$ were found to be unstable and appeared to promote cell lysis.

HrpZ purification and analysis.

HrpZ was purified from *E. coli* as previously described (He et al. 1993) with the following modifications. Cells were lysed in either 5 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.5, or cell lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0). For some experiments the supernatant from heat-treated lysate was partially purified after sonication by ammonium sulphate precipitation (25 to 45% saturation), with desalting and concentration being performed with Centricon-10 tubes (Amicon). For experiments requiring highly purified HrpZ expressed in *E. coli* BL21(DE3), the supernatant was further purified by binding to phenyl-sepharose (Sigma) in the presence of ammonium sulphate (>30% saturation) and elution with 5 mM MES, pH 5.5, followed by electrophoresis through a native 15% polyacrylamide gel. The purified protein was then eluted from excised gel slices using an Elutrap apparatus (Schleicher & Schuell) or from crushed gel slices using a Micropure separator (Amicon). Protein concentrations were determined using Bio-Rad protein assay solution. HrpZ was also purified from heat-treated supernatants of *P. syringae* grown in *hrp*-inducing medium (Huynh et al. 1989) by ammonium sulphate precipitation (25 to 45% saturation) and desalting/concentration using Centricon-10 tubes. For infiltration into plant tissue, HrpZ preparations were diluted to various degrees with 5mM MES, pH 5.5. The amino-terminal sequence analyses were performed at the Cornell Biotechnology Program Protein Analysis Facility ($HrpZ_{Pst}$) and the University of Kentucky Macromolecule Structure Analysis Facility ($HrpZ_{Pst}$).

T7 expression and labeling of proteins in *E. coli*.

Proteins encoded by the *hrpZ* operon were expressed in *E. coli* BL21(DE3) by using the pET21(+) T7 expression system (Novagen). Conditions for isopropyl-β-D-thiogalactopyranoside (IPTG) induction of T7 RNA polymerase-dependent expression and labeling with L-[^{35}S]methionine were as described by Studier et al. (1990). After being labeled, cells were collected by centrifugation and then resuspended and lysed in SDS-loading buffer and the proteins resolved on an SDS-polyacrylamide gel. Gels were stained, dried and exposed to Kodak X-ray film.

Nucleotide sequence accession numbers.

The nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers L41861 (*P. syringae* pv. *tomato hrpA, hrpZ, hrpB, hrpC, hrpD, hrpE*), L41862 (*P. syringae* pv. *glycinea hrpA, hrpZ, hrpB*), L41863 (*P. syringae* pv. *syringae hrpA*), and L41864 (*P. syringae* pv. *syringae hrpB*).

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hrp Genes of Phytopathogenic Bacteria

U. Bonas

1 Introduction

In nature plants are resistant to the majority of pathogens, and many bacteria live in close contact with the plant without causing any harm (see chapter by Beattie and Lindow in this volume). Among the 1600 different species known in the bacterial kingdom only a small number (about 80) are plant pathogenic and in most cases highly specialized with respect to the plant that can be attacked. Only a few of these species are gram-positive, e.g., *Clavibacter* ssp. and *Streptomyces* ssp. In this review I focus on subspecies of the gram-negative genera *Erwinia*, *Pseudomonas*, and *Xanthomonas*, which comprise the major bacterial plant pathogens.

To be a successful pathogen the invading bacterium has to overcome the plant's defense. During evolution plant pathogenic bacteria have acquired multiple functions that enable them to colonize and multiply in living plant tissue. In nature, bacteria enter the plant through natural openings (stomata, hydathodes) or

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wounds. The bacterial armory contains a number of weapons that contribute to pathogenicity. Obvious examples include degradative extracellular enzymes such as pectinases, cellulases, and proteases. When the corresponding genes are mutated, bacterial ability to invade plant tissues is more or less affected depending on the pathogen, i.e., these functions contribute to and modulate development and severity of infection to different extents (see chapters by Dow and Daniels, and Collmer and Bauer in this volume).

In addition, phytopathogenic bacteria possess a large number of genes needed for basic pathogenicity. These genes have been operationally defined as *hrp* (hypersensitive reaction and pathogenicity; LINDGREN et al. 1986) based on their mutant phenotype. *hrp* genes are not only essential for pathogenicity on a plant, i.e., the ability to cause disease in a compatible interaction, but also for the incompatible interaction with resistant host varieties or with plants that are not normally a host for the particular pathogen (so called non-host). The incompatible interaction is often associated with the induction of a hypersensitive reaction (HR) in the plant. In contrast to the use of the term hypersensitivity in the animal field, in plants the HR is a rapid defense response involving localized plant cell death, production of phenolics and antimicrobial agents, e.g., phytoalexins, at the site of infection (KLÉMENT 1982; LINDSAY et al. 1993). The HR results in prevention of pathogen multiplication and spread and thus in prevention of disease development. Under natural infection conditions the HR is microscopically small and can be induced by just one bacterial cell. Only when bacteria are introduced into plant tissue at high cell densities in the laboratory (about 10^7 colony forming units or more/ml) is the HR macroscopically visible as confluent necrosis and can be clearly distinguished from typical disease symptoms. It is important to note that saprophytic or nonpathogenic bacteria such as *Escherichia coli* or *Pseudomonas fluorescens* do not induce the HR and are unable to multiply in plant tissue.

2 Isolation of *hrp* Genes and General Features

hrp genes have been isolated from all major gram-negative plant pathogenic bacteria except Agrobacterium. There are excellent reviews that describe the early work or focus more on one particular pathogen (WILLIS et al. 1991; BOUCHER et al. 1992). The majority of *hrp* genes have been identified by complementation of loss-of-function mutants. Mutants obtained by random chemical (e.g., N-methyl-*N'*-nitro-*N*-nitrosoguanidine) or transposon mutagenesis of a pathogenic wild-type strain were inoculated into the host plant and screened for loss of both the ability to cause disease in susceptible plants and to induce the HR in resistant host or non-host plants (often tobacco). The second criterion for the isolation of genes specific for the plant interaction was to ensure that the mutants would still grow in minimal medium. This way mutants affected in genes for basic housekeeping functions were eliminated. A third characteristic of all *hrp* mutants is that they are unable to grow in the plant.

The *hrp* genes were originally described for the bean pathogen *Pseudomonas syringae* pv. *phaseolicola*. LINDGREN and coworkers (1986) isolated Tn*5*-induced mutants of *P.s.* pv. *phaseolicola* that had lost both the ability to induce halo-blight disease on bean and the HR in tobacco. Complementation with cosmid clones from a genomic library of the wild-type strain resulted in isolation of a cluster of *hrp* genes localized in a 20 kb DNA region. This was the first indication that both the ability to cause disease and to induce the HR are mediated by common steps in a "pathway".

Since then *hrp* gene clusters have been cloned from a number of different bacteria. Examples include *Pseudomonas solanacearum* (BOUCHER et al. 1987; Fig. 1B), the *Xanthomonas campestris* pathovars *campestris* and *vitians* (ARLAT et al. 1991), *translucens* (WANEY et al. 1991), and *vesicatoria* (BONAS et al. 1991; Fig. 1A), *Erwinia amylovora* (STEINBERGER and BEER 1988; BARNY et al. 1990; WALTERS et al. 1990; BAUER and BEER 1991), and several other pathovars of *P. syringae* (e.g., HUANG et al. 1988; LINDGREN et al. 1988; Fig. 1C). In addition, genes with DNA homology, and in some cases functional homology, have been isolated from other species, e.g., the so-called *wts* genes from *E. stewartii* (COPLIN et al. 1992; LABY and BEER 1992), and a region containing pathogenicity genes from *X.c.* pv. *glycines* that complement *hrp* mutants of *X.c.* pv. *vesicatoria* (HWANG et al. 1992; Bonas, unpublished results). Interestingly, nonpathogenic xanthomonads that were originally isolated from diseased plants as opportunists together with pathogenic bacteria do not contain *hrp*-related DNA sequences (STALL and MINSAVAGE 1990; BONAS et al. 1991). In *Agrobacterium tumefaciens* or in strains of *Rhizobium* ssp. there seem to be no *hrp* gene equivalents present (BONAS et al. 1991; LABY and BEER 1992). This conclusion is based on DNA hybridization experiments and, of course, does not exclude the presence of genes with functional homology to *hrp* genes in these species.

In all of the cases mentioned above, the *hrp* genes are organized in clusters of 22–40 kb, and I will restrict most of this chapter to these large *hrp* clusters. In addition, several smaller *hrp* loci have been described that are not linked to the large cluster present in the same bacterium. These include a region in *P. solanacearum* (HUANG et al. 1990), the *hrpX* locus that is conserved in *X. campestris* pathovars *campestris* (KAMOUN and KADO 1990; KAMOUN et al. 1992) and *oryzae* (KAMDAR et al. 1993), and the *hrpM* locus in *P.s.* pv. *syringae* (NIEPOLD et al. 1985; MUKHOPADHYAY et al. 1988). *hrpM* is functionally conserved in pathovar *phaseolicola* (FELLAY et al. 1991). Besides being nonpathogenic and unable to induce the HR in tobacco, *P. syringae hrpM* mutuants are also affected in mucus production. The *hrpM* locus encodes two putative proteins which are similar and have been shown to be functionally homologous to the products of the *E. coli mdoGH* operon (LOUBENS et al. 1993). The *mdoGH* genes are required for periplasmic membrane-derived oligosaccharide synthesis in *E. coli*. The *hrpQ* and *hrpT* genes from *P.s.* pv. *phaseolicola* (MILLER et al. 1993) will be discussed later in this chapter.

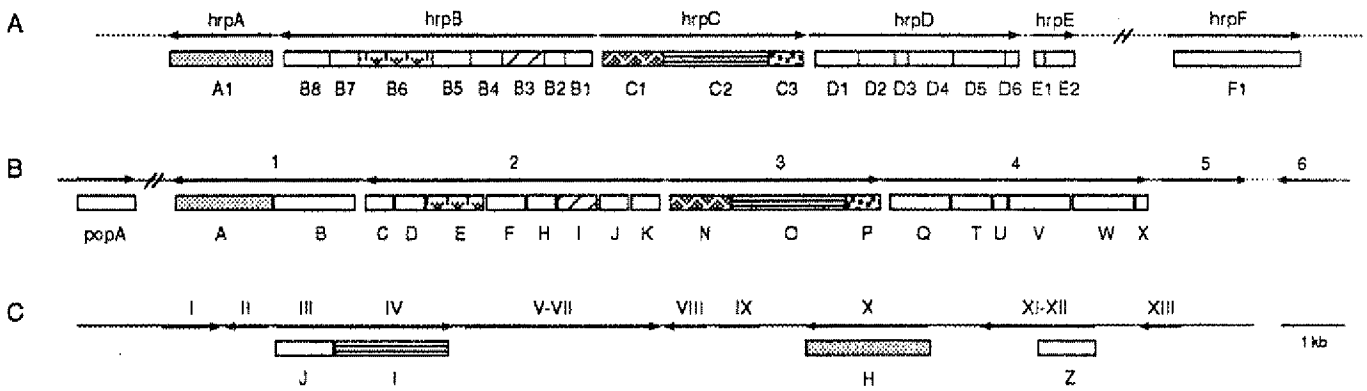

Fig. 1A–C. Genetic and translational organization of the *hrp* gene cluster of different plant pathogenic bacteria. **A** *Xanthomonas campestris* pv. *vesicatoria*; **B** *Pseudomonas solanacearum*; and **C** *Pseudomonas syringae* pv. *syringae*. Arrows represent transcription units as determined by genetic analyses. Boxes correspond to sequences of open reading frames (ORFs) that have been published. In case of sequence similarities between ORFs in different clusters the boxes are filled with the same pattern. For references, see text

3 Structural Organization and Relatedness of *hrp* Clusters

Genetic studies using transposon-induced insertion mutants in the respective bacterial wild-type strains revealed that the *hrp* clusters contain at least six to eight complementation groups (Fig. 1). Some *hrp* gene clusters have clearly been shown to be localized in the chromosome, e.g., in *P.s.* pv. *phaseolicola* (Rahme et al. 1991) and in *X.c.* pv. *vesicatoria* (Bonas et al. 1991), whereas in *P. solanacearum*, the *hrp* cluster is on a megaplasmid (Boucher et al. 1987).

Striking similarities have recently been found between the *hrp* genes of pathogens belonging to different genera. The first indication of homologies came from Southern hybridization studies. DNA homology was observed among different strains of the same pathovar, as well as between pathovars or strains within a species, and in some cases also between species. However, the degree of conservation varies. DNA homology is high within pathovars of a given subspecies, e.g., in *P. syringae* (Lindgren et al. 1988; Huang et al. 1991) and in *X. campestris* (Bonas et al. 1991). The latter studies were recently extended by PCR using primers based on *hrp* sequences from *X.c.* pv. *vesicatoria* (Leite et al. 1994). Furthermore, at least some regions of the *hrp* clusters appear to be conserved on the DNA level between *P. solanacearum* and pathovars of *X. campestris*, *P. syringae*, and also to *E. amylovora* (Boucher et al. 1987; Arlat et al. 1991; Gough et al. 1992; Laby and Beer 1992). In addition, cross-complementation within a subspecies indicated a high degree of functional conservation of *hrp* genes (e.g., Lindgren et al. 1988; Arlat et al. 1991; Bonas et al. 1991; Laby and Beer 1992). Due to sequence data it is now becoming more and more apparent that several *hrp* genes are conserved in all major gram-negative plant pathogenic bacteria (see below). Whether there are *hrp* genes that are clearly pathovar-specific can only be answered when complete sequence information becomes available for several *hrp* clusters.

4 Function of *hrp* Genes in *Xanthomonas campestris* pv. *vesicatoria* and Other Plant Pathogenic Bacteria

DNA sequence analysis of the *hrp* genes has revealed some important clues to their possible biochemical functions. One of the first genes sequenced was a regulatory gene, *hrpS*, from *P.s.* pv. *phaseolicola* (Grimm and Panopoulos 1989). This gene as well as *hrpB*, a regulatory gene from *P. solanacearum* (Genin et al. 1992), will be discussed below in the context of gene regulation.

Since *hrp* genes are environmentally regulated (see below), it was believed for a while that they would be encoding "alternative" proteins required for adaptation of the bacterium to the plant as the preferred environment. The recently discovered sequence similarities between several putative Hrp proteins and known proteins from other bacteria, however, led to a very different hypothesis, namely, involvement of Hrp proteins in protein secretion. We have

sequenced the entire *hrp* cluster of *X.c.* pv. *vesicatoria*. Since most *hrp* sequences from this and other bacteria are not yet published, I will summarize our results and refer to the other phytopathogenic bacteria as I go along. Based on genetic analyses and the open reading frames (ORFs) with a high coding probability we predict 21 *hrp* genes in the 25 kb *hrp* cluster of *X.c.* pv. *vesicatoria*. Their transcriptional organization is depicted in Fig.1A. The loci *hrpA* and *hrpB* are transcribed from right to left; the other four loci are transcribed from left to right (SCHULTE and BONAS 1992a). According to the locus (*hrpA–hrpF*) we have numbered the ORFs consecutively. The *hrpA* locus appears to contain just one *hrp* gene, *hrpA1*. The *hrpB* operon contains eight ORFs, called *hrpB1–hrpB8*, etc. A region of about 4 kb between *hrpE* and *hrpF* does not seem to be involved in the interaction with the plant because insertions in this region do not lead to a change in phenotype (BONAS et al. 1991).

What are the characteristics of the Hrp proteins? It should be noted that, except for three proteins, expression of the other 18 has yet to be demonstrated in *X.c.* pv. *vesicatoria*. A number of putative Hrp proteins are most likely associated with or localized in the bacterial membrane. For example, the HrpC2 protein sequence contains eight transmembrane domains but lacks a signal sequence, suggesting an inner membrane localization (FENSELAU et al. 1992). Both HrpA1 and HrpB3 contain an NH_2-terminal signal sequence and one (HrpA1) or two (HrpB3) transmembrane domains, suggesting that a part of these proteins might be targeted to the outer membrane. The signal sequence of HrpB3 resembles signal peptidase II sequences which are typical of lipoproteins (FENSELAU et al. 1992). Experiments using radioactively labeled palmitate are underway to test whether HrpB3 is a lipoprotein. Recently, both HrpB3 and HrpA1 were shown to be localized in the *X.c.* pv. *vesicatoria* membrane fraction using polyclonal antibodies (S. Fenselau, C. Marie, and U. Bonas, manuscript in preparation). The HrpB6 protein is a putative ATPase with highly conserved nucleotide and magnesium binding domains. It is more similar to protein traffic ATPases than to proton pump ATPases, and the lack of membrane spanning domains suggests a cytoplasmic location (FENSELAU et al. 1992).

Searches of the database revealed sequence relatedness of more than half of the *X.c.* pv. *vesicatoria* Hrp proteins with putative proteins in other bacteria, including different plant pathogens. High DNA sequence identity (more than 90%) was found to a 2.7 kb fragment carrying pathogenicity genes from *X.c.* pv. *glycines* (HWANG et al. 1992). The authors predicted two ORFs, whereas in *X.c.* pv. *vesicatoria*, this region contains three ORFs corresponding to the *hrpC3, hrpD1* and *hrpD2* genes. Complementation studies indicated that part of the *hrp* region is colinear in the two pathovars of *Xanthomonas* (unpublished).

The deduced amino acid sequences of *hrp* genes published from *P. solanacearum* (GOUGH et al. 1992, 1993; GENIN et al. 1992) show significant similarity to *X.c.* pv. *vesicatoria* proteins (Table 1; Fig.1). One exception is the *hrpB* regulatory gene from *P. solanacearum* which is not present in the 25 kb *hrp* region or in the flanking region of the *X.c.* pv. *vesicatoria hrp* cluster as determined by DNA sequence analysis and hybridization studies (T. Horns and U. Bonas,

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tudies (T. Horns and U. Bonas,

unpublished). Furthermore, several of the proteins mentioned are conserved in other species (Fig.1), however, the degree of sequence similarity varies greatly (Table 1). The HrpA1 protein from *X.c.* pv. *vesicatoria* is 48% and 29% identical to proteins from *P. solanacearum* (HrpA; Gough et al. 1992) and *P.s.* pv. *syringae* (HrpH; Huang et al. 1992), respectively. HrpC2 from *X.c.* pv. *vesicatoria* is even more conserved, being 66% idential to the corresponding HrpO protein of *P. solanacearum* (Gough et al. 1993), whereas the *hrpI* genes from *E. amylovora* (Wei and Beer 1994) and from *P.s.* pv. *syringae* (Huang et al. 1993) both show 62% similarity to *hrpC2* from *X.c.* pv. *vesicatoria*. *P.s.* pv. *syringae* also contains a *hrpB3* related gene, called *hrpY*, and a *hrpD2* related gene, *hrpW* (H.-C. Huang, personal communication). Thus, the high degree of DNA sequence conservation that was reported earlier (see above) is also seen on the protein level. It appears that *hrp* genes in *X.c.* pv. *vesicatoria* are more closely related to *P. solanacearum* than to *P. syringae* and to *Erwinia*. As more and more homologous *hrp* genes are found in other bacteria nomenclature might become confusing. However, as long as the genes have not been shown to be functionally homologous, we will continue to use these names.

Besides genes that are conserved among the major phytopathogenic bacteria some genes are absent in the *hrp* region of more distantly related species. For example, there are no known homologs of the harpin genes *hrpN* (Wei et al. 1992a), and *hrpZ* (He et al. 1993) (see below), and of *hrpJ* from *P.s.* pv. *syringae* (Huang et al. 1993) in the *X.c.* pv. *vesicatoria hrp* cluster (unpublished; see Fig. 1).

Similarities of 50%–60% were found recently between HrpA1 and HrpB3 from *X.c.* pv. *vesicatoria* and two putative NolI proteins of *Rhizobium fredii* that are encoded by a cultivar specificity region. NolT and NolW mutants have a wider host range in nodulation of soybean (Meinhardt et al. 1993). In addition, the authors mention that release of proteins is affected.

Last but not least, Table 1 summarizes the significant sequence similarities which have been found to proteins from animal bacterial pathogens. A number of putative Hrp proteins are related to proteins in animal pathogens such as *Salmonella, Shigella*, and *Yersinia* ssp. Since the first similarities found were to the Ysc, Vir, and Lcr proteins from *Yersinia* ssp, this group of organisms became a "role model" for plant pathologists (Fenselau et al. 1992; Gough et al. 1992; Huang et al.1992). In *Yersinia*, these proteins are essential for the secretion of virulence factors, called Yops (Yersinia outer protein; Michiels et al. 1990, 1991). Since they are described in detail in the chapter by G.R. Cornelis, I will mention only a few important features. The Yops are hydrophilic proteins that lack a typical NH_2-terminal signal peptide, and are secreted by using an entirely different pathway from that previously described for protein secretion. The genes involved in secretion are clustered on a 70 kb virulence plasmid. In case of a mutation, e.g., in *Yscj*, the Yops accumulate in the cytoplasm (Michiels et al. 1991). Although their direct role in transport has yet to be demonstrated, it is believed that the Ysc and Lcr proteins mentioned in Table 1 are parts of a special transport apparatus for Yop secretion. Similarly, *Shigella flexneri* secretes virulence factors, called Ipa (invasion plasmid antigens), that are distinct from Yops but share the general

Table 1. Sequence similarities of *Xanthomonas campestris* pv. *vesicatoria* Hrp proteins

Xanthomonas campestris pv. *vesicatoria*	HrpA1[1]	HrpB6[1]	HrpB3[1]	HrpC1[2]	HrpC2[1]	HrpC3[2]	HrpD1[2]	HrpD2[2]
Pseudomonas solanacearum	HrpA[3] (66%)	HrpE[3]	HrpI[3] (70%)	HrpN[5] (74%)	HrpO[3] (81%)	HpaP[5] (54%)	HrpO[4]	HrpT[4]
Pseudomonas syringae pv. *syringae*	HrpH[3] (52%)				Hrp1[7] (62%)			
Yersinia enterocolitica	YscC[8] (55%)		YscJ[8] (56%)					
Yersinia pestis	YscC[9] (55%)				LcrD[10] (70%)		LsaA[11] (52%)	LsaB[11] (72%)
Yersinia pseudotuberculosis		YscN[12] (73%)	LcrKa[13] (56%)					
Shigella flexneri	MxiD[14] (50%)	Spa47[15] (65%)	MxiJ[16] (52%)	Spa40[17] (55%)	MxiA[18] (65%)			Spa24[19] (67%)
Salmonella typhimurium	InvG[18] (52%)	SpaL[20] (70%) FliI[21] (65%)		SpaS[20] (56%)	InvA[22] (67%)			SpaP[20] (64%)
Bacillus subtilis		FlaA-ORF4[23] (68%)		FlhB[24] (62%)	FlhA[25] (63%)			FliP[26] (68%)
Escherichia coli		β-F1[27] (53%)						FliP[28] (65%)
Erwinia carotovora							MopB[29] (49%)	MopC[29] (65%)
Erwinia amylovora					Hrp1[30] (62%)			
Rhizobium fredii	NolW[31] (51%)		NolT[31] (61%)					
Caulobacter crescentus					FlbF[32] (55%)			

Bacillus subtilis		FliA-ORF4[27] (68%)		HrcB[28] (62%)	HrcA[29] (63%)		FliP[26] (68%)
Escherichia coli		β-F1[27] (53%)					FliP[28] (65%)
Erwinia carotovora						MopB[29] (49%)	MopC[28] (65%)
Erwinia amylovora					HrpI[30] (62%)		
Rhizobium fredii	NolW[31] (51%)		NolT[31] (61%)				
Caulobacter crescentus					FlbF[32] (55%)		

Similarities between deduced amino acid sequences of Hrp proteins from *X.c.*pv.*vesicatoria* and other proteins include conservative amino acid exchanges. Number in parentheses indicates percent similarity.
Superscript numbers indicate references as follows:
1, FENSELAU et al. 1992; 2, Bones et al., unpublished; 3, GOUGH et al. 1992; 4, GENIN et al. 1993, sequences unpublished; 5, GOUGH et al. 1993; 6, HUANG et al. 1992; 7, HUANG et al. 1993; 8, MICHIELS et al. 1991; 9, HADDIX and STRALEY 1992; 10, PLANO et al. 1991; 11, Fields et al. unpublished, accession # L22495; 12, Galyov, unpublished, accession # U00998; 13, RIMPILAINEN et al. 1992; 14, ALLAOUI et al. 1993; 15, VENKATESAN et al. 1992; 16, ALLAOUI et al. 1992; 17, SASAKAWA et al. 1993; 18, ANDREWS and MAURELLI 1992; 19, Lodge et al., unpublished, accession # X75302; 20, GROISMAN and OCHMAN 1993; 21, VOGLER et al. 1991; 22, GALÁN et al. 1992; 23, ALBERTINI et al. 1991; 24, Carpenter et al., unpublished, accession # X741212; 25, CARPENTER and ORDAL 1993; 26, BISCHOFF et al. 1992; 27, SARASTE et al. 1981; 28, MALAKOOTI et al. unpublished, accession # L21994; 29, MULHOLLAND et al. 1993; 30, WEI and BEER 1993; 31, MEINHARDT et al. 1993; 32, RAMAKRISHANAN et al. 1991; SANDERS et al. 1992.

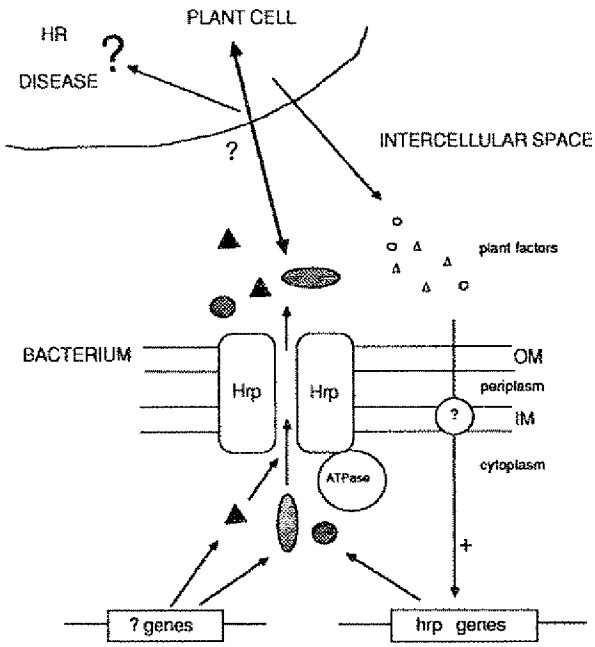

Fig. 2. Hypothetical model of cell signaling between gram-negative bacteria and plants indicating the proposed function of Hrp proteins as an apparatus for protein secretion. The model has been modified after FENSELAU et al. (1992). Hrp proteins may form a tunnel that enables the export of molecules such as virulence factors or avirluence factors leading to either a hypersensitive response (HR) or disease. These factors could be encoded by *hrp* genes or genes unlinked to the large cluster. Both types of genes have been found to encode elicitors of the HR (see text). The secretion of virulence proteins is hypothetical

features mentioned above (HALE 1991; and see chapter by PARSOT, this volume). Although *S. typhimurium* appears to possess a secretion system similar to that in *Shigella*, secreted invasion antigens have not yet been identified (GROISMAN and OCHMAN 1993; see chapter by FINLAY). As unpublished reports indicate that more and more genes in the animal pathogens are conserved, the data shown in Table 1 will soon be out of date. Proteins from other bacteria, e.g., *E. coli, Bacillus, Caulobacter* and from the *mop* region in *E. carotovora* (MULHOLLAND et al. 1993), have also been found to be similar to Hrp proteins (Table 1). Most of these are important for the assembly of the flagella, motility, or chemotaxis, again pointing, in my opinion, to a specialized secretion system rather than an involvement of *hrp* genes in chemotaxis.

These observations led us and others to propose a *hrp*-dependent secretion system in plant pathogenic bacteria (FENSELAU et al. 1992; GOUGH et al. 1992; VAN GIJSEGEM et al. 1993). A model is shown in Fig. 2 and raises certain questions, e.g., if secretion occurs, what is being secreted by plant pathogenic bacteria? So far, a few proteins have been identified as elicitors of the HR but there is no evidence for secretion of virulence factors (see below).

5 *hrp*-dependent Secretion of Hypersensitive Response-Inducing Proteins

5.1 Harpin from *Erwinia amylovora*

An important feature of the isolated *hrp* clusters from both *E. amylovora* and *P.s.* pv. *syringae* is the ability of *E. coli* or *Pseudomonas fluorescens* transformants containing the cloned genes to induce the HR on tobacco (Huang et al. 1988; Beer et al. 1991; see below). This has prompted to search for the HR-inducing activity within the respective gene clusters.

The first bacterial HR-inducing protein identified, designated harpin, is a cell envelope-associated protein encoded by the *hrpN* gene of *E. amylovora*, a pathogen of pear and apple (Wei et al. 1992a). This harpin$_{Ea}$ is a glycine-rich and heat-stable protein that induces the HR in the non-host, tobacco. The *hrpN* gene is localized within the respective *hrp* cluster and thus has a dual role in also being required for pathogenicity on the normal host plant. Its function in pathogenicity, however, is unknown. Beer et al. (1993) mentioned in a preliminary report that the *hrpN* gene seems to be conserved among *Erwinia* ssp. but that there is no DNA homology between *hrpN* and sequences in the other plant pathogenic bacteria. Although data described below suggest that the harpin$_{Ea}$ protein might be secreted via the Hrp secretory apparatus, there is no published information available that demonstrates this.

5.2 Harpin from *Pseudomonas syringae* pv. *syringae*

Using an elegant approach He and coworkers recently have identified harpin$_{Pss}$, which is encoded by the *hrpZ* gene in the bean pathogen *P.s.* pv. *syringae* (He et al. 1993; see Fig. 1C and chapter by Collmer and Bauer). Lysates of *E. coli* clones containing an expression library, made using the cloned *P.s.* pv. *syringae hrp* cluster, were directly screened for HR-inducing activity on tobacco leaves. Two proteins were identified, one of which was an NH_2-terminal deletion of harpin$_{Pss}$ with even higher activity than the full size protein. Whether or not processing occurs in natural infection is not clear. Interestingly, two short direct repeats in the COOH-terminus of harpin$_{Pss}$ are essential for elicitor activity. Although the two harpins harpin$_{Ea}$ and harpin$_{Pss}$ differ in their primary sequence, they have several features in common, e.g., a stretch of 22 amino acid that is similar in both proteins (He et al. 1993). Harpin$_{Pss}$ is also glycine-rich and heat-stable. As with harpin$_{Ea}$ of *E. amylovora*, the function of harpin$_{Pss}$ in pathogenicity is unknown. Its product is secreted by *P.s.* pv. *syringae* in a HrpH-dependent way; HrpH is highly related to proteins involved in secretion in other plant and animal pathogens (Huang et al. 1992; see Table 1).

5.3 *PopA* from *Pseudomonas solanacearum*

An HR-inducing protein has been identified and characterized from *P. solanacearum* culture supernatants, called Pop (Pseudomonas out protein; ARLAT et al. 1994). PopA1 and two shorter derivatives, PopA2 and PopA3, induce the HR in tobacco and in certain, but not all, *Petunia* lines. Like the harpins, the Pop proteins are also heat-stable and glycine-rich, however, the sequence is entirely different. In contrast to the harpins, the *popA* gene is not a *hrp* gene but is located outside of the large *hrp* cluster. Interestingly, expression of *popA* is *hrpB*-dependent, i.e., the gene is part of the *hrp* regulon. Mutations in *popA* do not affect the HR on tobacco or pathogenicity on tomato suggesting that more than one HR-inducing factor is produced. ARLAT et al. (1994) convincingly showed that secretion of PopA is dependent on other *hrp* genes, such as *hrpA*, *hrpN*, and *hrpO* (Fig. 1B). If a bacterial strain virulent towards *Petunia* is found it will be interesting to see if PopA acts as an avirulence protein in *Petunia* as has been suggested by the authors.

These exciting findings prove that certain Hrp proteins of *P.s.* pv. *syringae* and *P. solanacearum* play a role in transport of HR elicitors (Fig. 2). They also stimulate more questions. It needs to be shown that harpins and PopA are in fact secreted when the bacteria interact with the plant (the *hrp* genes were induced in vitro). Are harpins conserved among pathovars of *P. syringae*? How many elicitors of the non-host HR in tobacco can be found? Is the mechanism of recognition in tobacco identical with the *Erwinia* and *P.s.* pv. *syringae* harpins and the *P. solanacearum* Pops?

6 Regulation of Expression of *hrp* Genes

Expression of *hrp* genes is controlled by environmental conditions and has been studied on the RNA level as well as using transcriptional fusions to reporter genes such as the *E. coli* genes encoding β-galactosidase or β-glucuronidase. In general, expression of *hrp* loci is not detectable when the bacteria are grown in complex culture media. However, after growth of the bacteria in the plant, *hrp* genes are expressed. Attempts to mimic the conditions that the different bacterial species encounter in the plant tissue resulted in the finding that growth in minimal media without any plant-derived factor was sufficient to induce *hrp* genes. This has led to the speculation that the bacteria have to experience some kind of starvation conditions for full expression of *hrp* genes. One of the first indications for *hrp* gene expression in vitro, and clearly a breakthrough, was a report on the *hrp*-dependent expression of an avirulence gene from the soybean pathogen *P.s.* pv. *glycinea* (HUYNH et al. 1989).

Since the composition of minimal media differs depending on the bacterium studied, the most important findings will be summarized for representative pathogens. Parameters like carbon source, concentration of organic nitrogen and phosphate, osmolarity, and pH have been found to be important. High con-

centration of organic nitrogen generally appears to suppress *hrp* gene activation. Only two regulatory genes have been studied so far (see below). Interestingly, they both belong to different families of regulatory proteins.

6.1 *Pseudomonas syringae*

Expression of all seven *hrp* loci in the large cluster of *P.s.* pv. *phaseolicola* is suppressed in complex medium but induced in the plant. Induction occurs in the susceptible host plant as well as in the non-host, tobacco, suggesting that there is no plant species-specific molecule involved in control of host range (RAHME et al. 1992). Five complementation groups, *hrpAB, hrpC, hrpD,* hrpE and *hrpF,* can also be induced in M9 minimal medium containing sucrose as a carbon source, however, induction is affected by pH, osmolarity, and carbon source, and never reaches the levels obtained in the plant (RAHME et al. 1992). A similar observation was made earlier for the avirulence gene *avrB* in *P.s.* pv. *glycinea.* Induction occurred in a minimal medium containing fructose, mannitol, or sucrose. Expression of *avrB* is dependent on *hrp* genes homologous to *hrpRS* and *hrpL* from *P.s.* pv. *phaseolicola* and was suppressed by TCA cycle intermediates such as citrate and succinate (HUYNH et al. 1989). *hrp* gene expression in *P.s.* pv. *syringae* occurs in the same medium as described by HUYNH et al. (1989); (HUANG et al. 1991; XIAO et al. 1992). The authors showed *hrp* gene induction in the non-host plant, tobacco, but no data for the host plant. The *P.s.* pv. *phaseolicola* loci *hrpL* and *hrpRS* are only expressed to a very low level in M9 minimal medium and are induced at least 1000-fold when the bacteria are inoculated into the plant. This led to the conclusion that, at least for expression of *hrpL* and *hrpRS,* specific plant factors might be necessary (RAHME et al. 1992).

6.2 Regulatory Genes *hrpRS* and *rpoN* of *Pseudomonas syringae* pv. *phaseolicola*

The results on environmental factors inducing or suppressing *hrp* gene expression suggested that specific regulatory genes are involved in the control of *hrp* promoter activities. At least two loci are involved in positive regulation of the other *hrp* loci of *P.s.* pv. *phaseolicola hrp* cluster (FELLAY et al. 1991). While there is no information published for *hrpL, hrpRS* has been sequenced. It contains two genes whose predicted protein products are 60% identical to each other (GRIMM and PANOPOULOS 1989; MILLER et al. 1993). The HrpS protein is similar to members of the NtrC family of regulatory proteins in gram-negative bacteria. Most NtrC-like regulatory proteins are members of two-component systems, with a sensor protein that in turn activates a response element by phosphorylation of a site in the conserved NH_2-terminal domain (ALBRIGHT et al. 1989). The putative sensor component operating in *hrp* gene regulation has not been identified. It is postulated that HrpS is the activating protein, however, direct biochemical data

have not been presented. The lack of a typical NH_2-terminal domain in HrpS could indicate that a different mechanism may be involved in HrpS activation. Apparently, *hrpS*-related sequences are also present in other bacteria, e.g., in *P.s.* pv. *syringae* (HEU and HUTCHESON 1993) and in *Erwinia amylovora* (BEER et al. 1993). *E. stewartii* contains a transcriptional regulator, WtsA, with 52% identity to HrpS of *P.s.* pv. *phaseolicola*. The *hrpS* clone, however, was unable to functionally complement a *wtsA* mutant (FREDERICK et al. 1993).

The structure of the *hrpRS* locus and the finding of –24/–12 consensus sequences upstream of *hrpRS* indicated a possible role in transcriptional activation for transcription factor sigma 54, encoded by *rpoN* (GRIMM and PANOPOULOS 1989). In a preliminary report, FELLEY et al. (1991) demonstrated that *hrp* gene expression in *P.s.* pv. *phaseolicola* is indeed dependent on *rpoN*. A *rpoN* mutant of *P.s.* pv. *phaseolicola* is a glutamine auxotroph and nonpathogenic. Whether *rpoN* is generally involved in regulation of *hrp* gene expression is not clear. In *X.c.* pv. *vesicatoria*, *rpoN* is clearly not involved in *hrp* gene expression and pathogenicity (T. Horns and U. Bonas, manuscript in preparation).

Recently, MILLER et al. (1993) have reported the identification of two new loci, *hrpQ* and *hrpT*, from *P. s.* pv. *phaseolicola* that affect activation of *hrpRS* in *trans*. However, since *hrpRS* is strongly induced in plants while both *hrpQ* and *hrpT* are constitutively expressed, there must be more factors involved in *hrp* gene regulation. Strains carrying mutations in either *hrpQ* or *hrpT* are amino acid auxothrophs (methionine and tryptophan). *hrpQ* and *hrpT* are probably involved in methionine and tryptophan biosynthesis, respectively (MILLER et al. 1993). As stated above, such mutants would normally have been eliminated from the *hrp* mutant analysis.

6.3 Conserved Sequence Boxes in *Pseudomonas syringae*

A conserved sequence, the so-called harp box (TG(A/C)AANC, FELLAY et al. 1991), upstream of four *hrp* loci in *P. s.* pv. *phaseolicola*, was suggested to be involved in positive regulation of expression. Similar motifs were described for the promoter regions of several *P. syringae* avirulence genes, the expression of which is dependent on *hrpRS* and on *rpoN* (HUYNH et al. 1989; SALMERON and STASKAWICZ 1993; INNES et al. 1993; SHEN and KEEN 1993). These studies led to a revised 'harp' box sequence (GGAACCNA). Its significance in protein binding has not been shown but *avrD* promoter constructs lacking the harp box are no longer inducible (SHEN and KEEN 1993). A harp box-related motif was also found upstream of transcription unit 3 in *P. solanacearum* (GOUGH et al. 1993).

There is no harp box sequence in *Xanthomonas hrp* gene promoters. Another sequence motif that occurs in the promoter region of *hrp* loci in *X. c.* pv. *vesicatoria* was recently identified. This "PIP" (plant-inducible promoter) box has the sequence TTCGC-N15-TTCGC and occurs upstream of the –35 consensus sequence in four out of six *hrp* promoters (S. Fenselau and U. Bonas, unpublished). Experiments are underway to test whether this is a protein binding motif.

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6.4 *Xanthomonas campestris*

Expression of *hrp* genes in *X. c.* pv. *campestris* was determined after growth in vitro and found to be induced in a minimal medium with sucrose and/or fructose as carbon source. No expression occurred in complex media or with high concentrations of organic nitrogen (Arlat et al. 1991). In *X. c.* pv. *vesicatoria*, expression of the six *hrp* loci is induced in the plant but cannot be efficiently induced in the synthetic media tested so far. However, culture filtrates of sterile tomato cell suspension cultures (called TCM) induced expression of the six *hrp* loci in *X. c.* pv. *vesicatoria* whereas the basal Murashige-Skoog culture medium did not. The inducing factor(s) could only partially be purified from TCM and was found to be smaller than 1000 dalton, heat-stable, organic, and hydrophilic (Schulte and Bonas 1992a). De novo transcription of all *hrp* loci occurs rapidly within 1 h after transfer of the bacteria into TCM (S. Fenselau and U. Bonas, unpublished). A minimal medium was designed which would not suppress *hrp* gene induction. This medium, called XVM1, induces the *hrpF* locus (Fig. 1A) to high levels and differs from the other media described above, in particular by its low concentration in phosphate. Both sucrose and methionine are needed for efficient induction. It is not known whether a plant factor is necessary for activation of the other *hrp* loci, or if the XVM1 medium still lacks components or contains them in suppressing amounts (Schulte and Bonas 1992b).

6.5 *Erwinia* and *Pseudomonas solanacearum*

The *hrp* genes of *Erwinia amylovora* are rapidly induced in the non-host, tobacco, and more slowly in the host, pear. Several loci were induced in minimal medium with mannitol as a carbon source. Induction was suppressed by high concentrations of nitrogen and by glucose and was slightly temperature dependent (Wei et al. 1992b).

In *P. solanacearum*, the *hrp* cluster was also induced in host and in non-host plants, as well as in minimal medium. The best carbon sources for induction of four of the six transcription units were pyruvate and glutamate while, as in other bacteria, casamino acids suppressed induction (Arlat et al. 1992). The two rightmost *hrp* transcription units (5 and 6; Fig. 1B) are constitutively expressed but can be induced under certain conditions (Genin et al. 1992).

The only other gene reported to regulate *hrp* gene expression is *hrpB* from *P. solanacearum*. The gene is part of the *hrp* cluster and appears to be a member of the AraC family of positive regulatory proteins. Interestingly, *hrpB* is related to *virF* of *Yersinia* (Cornelis et al. 1989; Genin et al. 1992). The *hrpB* gene positively regulates four of the six *hrp* loci, as well as the *popA* locus, located outside of the *hrp* cluster which encodes a protein secreted in a Hrp-dependent way (see above; Arlat et al. 1994). Whether the HrpB protein binds directly to *hrp* promoters is not yet known.

At this time one can only speculate whether the regulatory systems for *hrp* gene expression employed by *P. solanacearum* and *P. syringae* are really different

or whether there is a global regulatory network thus allowing the fine tuning of gene expression in response to environmental cues. In conclusion, most *hrp* loci from different bacteria are inducible in a particular minimal medium. At this time it cannot be ruled out that stimulation of *hrp* gene expression involves specific plant factors as was described for the virulence genes of *Agrobacterium* (Winans 1992). Since the composition of the nutrients available to the pathogen in the plant is not known one can only speculate that the conditions described above reflect the situation in the plant. It is noteworthy that the in vitro culture will only mimic the dynamic nutritional situation that bacteria experience in their interaction with a plant for a short time. In mammalian bacterial pathogens, the expression of genes involved in virulence is also regulated in response to environmental cues rather than to specific host molecules. This subject has been reviewed recently (Mekalanos 1992 and in accompanying chapters), and I will only mention some important factors. In *Yersinia*, the *vir* and *lcr* genes are regulated by low calcium (low calcium response genes; Straley et al. 1993) and by temperature (Cornelis et al. 1989; see chapter by Cornelis). A calcium effect has not been described for any plant bacterium. In our laboratory no effect of calcium on *hrpF* gene expression in XVM1 was observed (Schulte and U. Bonas, unpublished). Expression of *invA* of *S. typhimurium* of the *mxi* and *ipa* genes of *Shigella* is affected by osmolarity and the later genes also by temperature (Galán and Curtiss 1990; Hale 1991).

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